



This number contains indices and completes Volume 45

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# Proceedings of the Society for Experimental Biology and Medicine

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11824

## Influence of Protamine Zinc Insulin upon Appetite During Anorexia of Vitamin B Deficiency.

RICHARD H. BARNES and EATON M. MCKENNA  
From The Staff, Medical College of Lehigh University

Protamine zinc insulin has a remarkable effect upon the appetite of normal animals, leading to a marked hyperalimentation and decrease in the excess of body fat. The influence of this insulin preparation upon the decreased appetite in the anorexia due to vitamin B deficiency was examined with the idea of improving

MCKENNA, E. M., and BARNES, R. H. *Proc. Soc. Exp. Biol. and Med.* 1941, 26, 44.  
BARNES, R. H., and MCKENNA, E. M. *Proc. Soc. Exp. Biol. and Med.* 1941, 26, 44.  
BARNES, R. H., and MCKENNA, E. M. *Proc. Soc. Exp. Biol. and Med.* 1941, 26, 44.



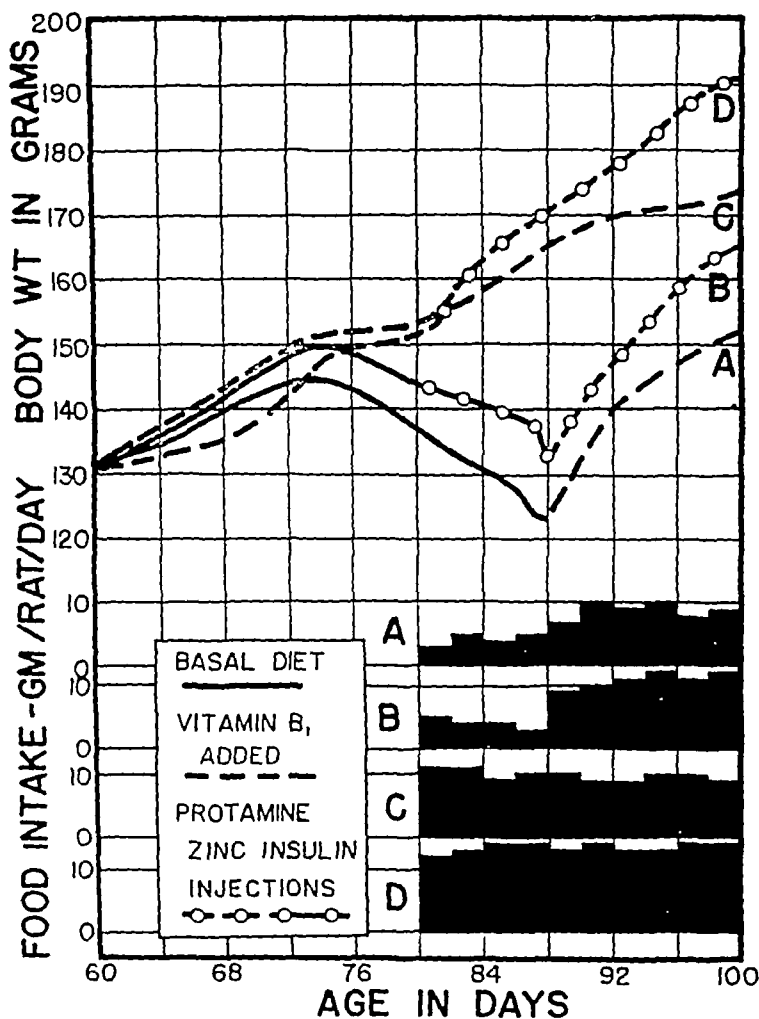


FIG 1

It is quite obvious from our experiments that vitamin B<sub>1</sub> in adequate amounts must be present in the organism if protamine zinc insulin is to lead to an increase in appetite. In normal rats we inclined toward the idea that the insulin hyperalimentation was a result of the depression of the blood sugar level.<sup>2</sup> Vitamin B<sub>1</sub>-deficient rats, however, died in hypoglycemia without any increase in the food intake whatever. Our diet was devised to study the conversion of carbohydrate to fat and so is practically fat-free.

more light on the mechanism of the insulin hyperalimentation in normal animals,<sup>2</sup> of gaining information relative to the reason for appetite failure when this vitamin is deficient and the question of the necessity of vitamin B<sub>1</sub> for the conversion of carbohydrate to fat.<sup>4, 5, 6</sup>

Four groups of six 60-day-old male rats were placed upon a diet\* composed of vitamin-free casein 20, sucrose 71, standard (Osborne and Mendel) salt mixture 4 and dried and powdered autoclaved brewer's yeast free of vitamin B<sub>1</sub> 5 parts. In addition there were 10 mg vitamin B<sub>6</sub>, 10 mg riboflavin and 20 mg nicotinic acid per 100 g of food. This diet was fed to groups A and B. Groups C and D were given the same diet with the addition of 0.5% thiamin chloride (Vit B<sub>1</sub>). Every second day each of the rats in all groups were given one drop of a fish liver oil containing 60,000 U S P Vit A units and 8500 U S P Vit D units per gram.

Figure 1 presents our experimental results. All of the groups gained well for some 12 days after which those (A and B) without the B<sub>1</sub> supplement commenced to lose weight. When the food intake was measured after 20 days the poor appetite of the B<sub>1</sub>-deficient rats was very obvious. At this time the administration of protamine zinc insulin (Squibb's product in a strength of 40 u per cc) was begun. Group D on an adequate diet were given 2 units subcutaneously morning and evening. Their food intake rapidly increased and their weight curve was obviously superior to their controls (Group C) on an adequate diet. A vitamin-deficient group which is not charted in Fig. 1 but which had a weight curve about like Group A were given the 2-unit insulin dose twice daily beginning at 81 days of age and all 6 rats in the group died in hypoglycemia within 3 days. Their appetite was not increased. The B<sub>1</sub>-deficient group B were therefore given 1 unit of insulin each twice daily. There was again no increased food intake and 3 of the 6 rats died in hypoglycemia before the end of the 8-day period (89 days old) when vitamin B<sub>1</sub> administration was commenced. At this time the rats in both groups A and B were given subcutaneously 5 mg thiamin chloride in aqueous solution every day. The food intake and body weight of both groups increased at once. The insulin-treated group did not do much better than the others until the dose of insulin was increased to 2 units twice daily when they were 93 days old.

<sup>4</sup> Whipple, D. V., and Church, D. F. *J. Biol. Chem.* 1936 **114**, cvii.

<sup>5</sup> Whipple, D. V., and Church, D. F. *J. Biol. Chem.* 1937, **119**, cv.

<sup>6</sup> McHenry, E. W., and Gavin, G. *J. Biol. Chem.* 1938 **125**, 653.

\* Their previous diet contained barely sufficient thiamin chloride for normal growth.

animals Recently Pecher<sup>2</sup> has demonstrated a marked similarity in the metabolism of radio-calcium and radio-strontium of mice and rats This suggested the use of radio-strontium instead of radio-calcium in determining the rate at which alkaline earth ions in the plasma are secreted into milk since  $^{90}\text{Sr}$  has a half life of 55 days and emits pure beta-particles at energies averaging about 750 000 electron volts At present there is no significant evidence that living cells can distinguish an element from its radioactive isotope To avoid the differences in the rates of absorption from the intestines between calcium and strontium, 50 cc of an isotonic solution of a mixture of inert and radioactive strontium lactate (850 mg of strontium) which emitted 11.2 microcuries of beta radiation per cc were given intravenously to each of 2 Holstein cows both in the fourth month of their lactation period and both producing about the same quantity of milk The milk was obtained hourly the first 6 hours after administration and twice a day for the following 4 days

Ten cc aliquots of each of the 28 samples were ashed at 400°C 100 cc portions of the second fourth seventh and ninth samples from each cow were placed in 400 cc of ethyl alcohol and agitated at irregular intervals for 10 days The samples were filtered and the filtrates and precipitates ashed separately 50 cc of the fifth sample of one cow was placed in a thick collodion bag on which 2 meters of water pressure was constantly applied for 72 hours The ultra-filtrate and the residue were ashed separately The various ashed samples were assayed for radioactivity on a standardized DuBridge electrometer and compared with aliquots of the dose of radio-strontium administered

CHART I

	Period after iv administration, hr	No cc's of milk secreted	% of dose of $^{90}\text{Sr}$ present in milk
Cow 227	0-6	2320	3.9
	6-30	8700	4.9
	30-54	8000	1.24
	54-78	7600	.54
	78-102	7700	.42
	Total	34355	11.09
Cow 259	0-6	1435	2.3
	6-30	6500	3.85
	30-54	7400	1.11
	54-78	6100	.43
	78-102	5400	.19
	Total	27135	7.58

<sup>2</sup> Pecher, Clara, PROC Soc EXP Biol AND MED, to be published.

Rats suffering from vitamin B<sub>1</sub> deficiency have an aversion to both carbohydrate and protein<sup>-</sup> and it is possible that insulin might influence the appetite for fat in B<sub>1</sub> deficiency but we have found the fat appetite less affected than that for carbohydrate in normal rats

*Summary* The administration of protamine zinc insulin which leads to a marked hyperalimentation in normal rats is without effect upon the depressed appetite of rats suffering from vitamin B<sub>1</sub> deficiency. These deficient rats succumb in hypoglycemia without an increase in their food intake when the insulin is administered. When thiamin chloride is given to B<sub>1</sub>-deficient rats their appetite responds not only to the vitamin therapy but to protamine zinc insulin as do normal animals

11825 P

### Secretion of Radio-Strontium in Milk of Two Cows Following Intravenous Administration \*

L. A. ERF,<sup>†</sup> AND CHARLES PECHER<sup>‡</sup> (Introduced by J. H. Lawrence)

*From Crocker Radiation Laboratory, University of California, Berkeley, California*

The composition of milk of cows varies markedly and is quickly influenced by the food ingested. To the pediatrician interested in infant nutrition this fact carries great significance. To determine, ultimately, the percentage of ingested calcium that is normally secreted in the milk of cows, the following preliminary experiment was made.

*Method* Harold Walke, *et al.*,<sup>1</sup> have shown that the only radio-calcium isotope suitable for biological investigation is <sup>45</sup>Ca. However, the poor yield of this isotope and the softness of its emitted radiations prohibit, at the present time, its use as a tracer in large

<sup>1</sup> Richter, C. P., Holt, L. E., Barellare, B., Jr., and Hawkes, C. D., *Am. J. Physiol.*, 1938, **124**, 596.

\* We wish to thank the crew managing the Berkeley cyclotron for producing the <sup>88</sup>Sr<sup>89</sup> used, and Dr. O. W. Schalm of the Department of Veterinary Science, University of California, who secured for us the cows used. The investigation reported here was aided by a grant from the John and Mary R. Markle Foundation for Medical Research.

<sup>†</sup> Wm. R. Kenan, Jr., Research Fellow

<sup>‡</sup> Fellow the Belgian American Education Foundation

<sup>1</sup> Walke, H., Thompson, F. C., and Holt, G., *Phys. Rev.*, 1940, **57**, 177

## 11826 P

Determination of Vitamin B<sub>1</sub> Requirement of Infants by Means of Urinary Excretion of Thiamin

ELIZABETH M. KNOTT (Introduced by Frederic W. Schlutz)

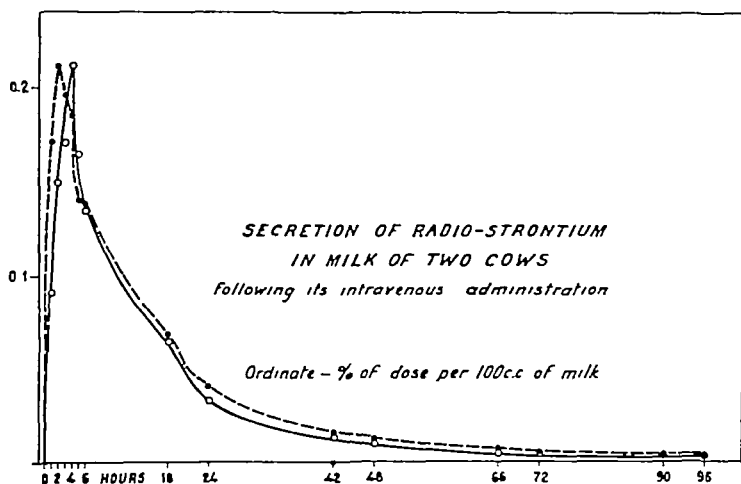
*From the Department of Pediatrics \* University of Chicago*

In order to determine the level of vitamin B<sub>1</sub> intake which will give optimum conditions for development for the young infant a series of 5-day balance studies have been conducted on each of 12 healthy male infants during the first 6 months of their lives. Thus far a total of 98 metabolism periods have been completed. The vitamin B<sub>1</sub> intake of the infants has been controlled either through the amount and type of milk fed each day or by the additions of pure thiamin to the formulas. The range of total intake has been from 21 to 313 units of thiamin per day. Periodically for each infant quantitative collections of urine and feces have been made for 5 consecutive days. These materials as well as the various milks have been assayed biologically for their thiamin content.

It was apparent early in the study that infants had absorbed thiamin from milk formulas very completely since only small quantities of the vitamin were excreted in the stools and in general the amounts of thiamin excreted in the urine increased with increases in intake. In the accompanying chart the amounts of urinary thiamin have been plotted in relation to the intake. A study of these plotted results reveals 2 shifts in the pattern of urinary excretion which seem significant. Below 80 units of intake per day a constantly low quantity of thiamin was excreted in the urine. At 140 units or more of intake the thiamin appeared to be excreted in much larger amounts as higher levels of the vitamin were fed. The average urinary thiamin for successive increments of 20 units of intake were as follows: 6.6, 6.4, 19.2, 22.3, 34.4, 47.1, 104.7, 77.6, 34.1, 118.8, 83.9, 96.6. The 2 bars are placed in the sequence at 80 and 140 units of intake per day. These data seem to indicate that 80 units may be the minimum requirement and 140 units the maximum amount needed for this age infant. Perhaps the range from 100 to 150 units of thiamin may be considered the optimum intake for a young healthy infant and that above this level of ingestion excess vitamin is excreted rapidly in the urine. The optimum for the baby however cannot be determined by economy of vitamin alone and infants may require a considerable continuous average

\* Assistance in the preparation of these materials was furnished by the Personnel of Works Project Administration Official Project No. 30230.





*Results* The accompanying graph and chart reveal that 11% of the dose of radio-strontium administered to the first cow was found in the 34 355 cc of milk secreted during the first 4¼ days, and 7 88% in the 27 135 cc of milk secreted by the second cow during the same period. The percentages of the dose found per 100 cc of milk from the two cows were almost identical (graph). The greatest amounts of radio-strontium were found in the milk during the first few hours following administration. This suggests that much of the calcium found in milk may be derived from that present in the diet, and Pecher has shown, in mice that some is derived from that most recently deposited in the trabecular portion of bones. 97-99% of  $^{89}\text{Sr}$  in all the samples studied was present in the precipitate of the alcohol-milk mixture and only 16% passed through the collodion bag as an ultra-filtrate. It is impossible to separate the calcium phosphate-calcium caseinate complex of milk and from the above findings it is clear that it is essentially impossible to separate the strontium complex also.

*Summary* 7 88% and 11% of a dose of radio-strontium were secreted in the milk of two Holstein cows during 4¼ days following its intravenous administration.

The usual method of administration consists of subcutaneous or intramuscular injections. It would be highly advantageous if one could administer estrogens in effective amounts without submitting the patient to the annoyance of repeated injections. Although it has been shown that estrogens are absorbed through the skin and the intestinal tract, neither method is satisfactory for therapeutic purposes.

In the present communication, we wish to describe a method for administering estrogens which is both simple and effective. It consists of sublingual administration of  $\alpha$ -estradiol in a propylene glycol solution.\* The solution contains 0.5 mg of  $\alpha$ -estradiol per cc of propylene glycol. Four drops are placed under the tongue and the patient is instructed not to swallow for five minutes. This can be done several times a day.

The present study was performed on a series of 8 women with

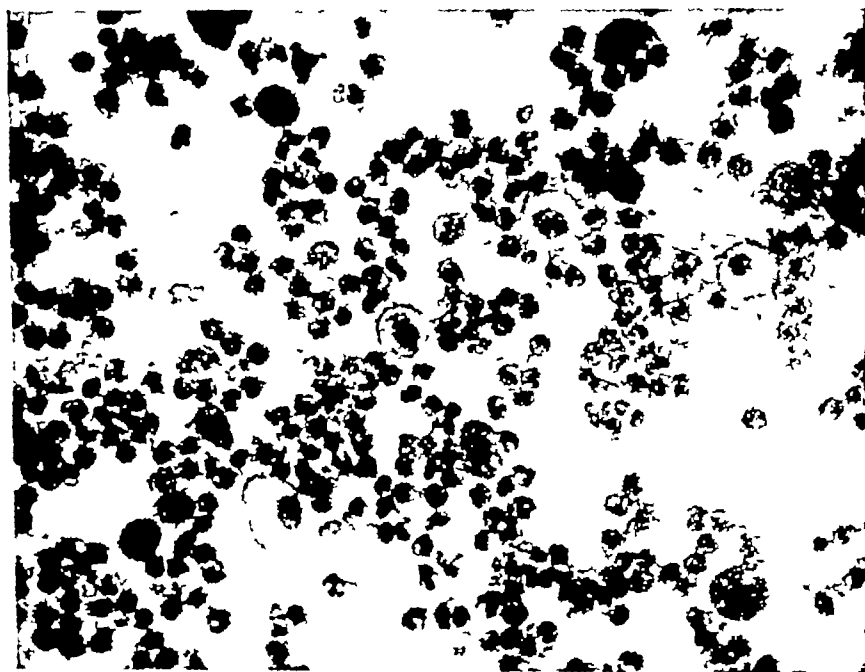
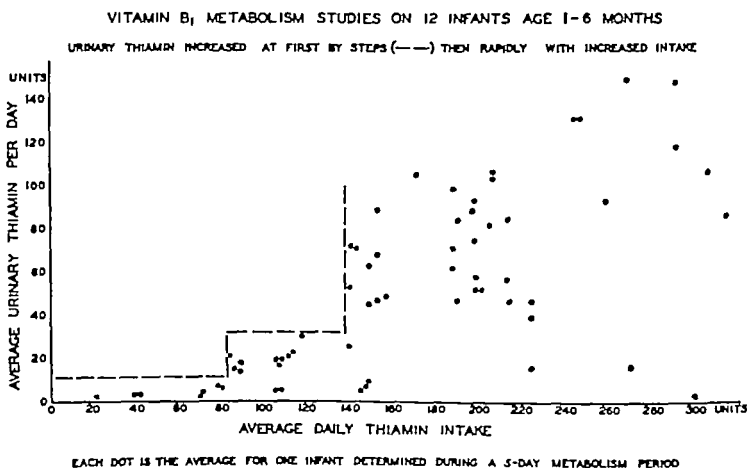


FIG 1

Patient SB, age 54, menopause 6 years ago. Pre-treatment vaginal smear revealing marked degree of estrogen deficiency.

\* For the materials used in this investigation, we are indebted to Dr. Edward Henderson, of the Schering Corporation, Bloomfield, N. J.



In order to determine thiamin requirements more exactly we have also studied the amounts of cocarboxylase found in the blood of young infants. Preliminary results show that at an intake increasing from 42 to 56 units of thiamin per day during 9 weeks of study, one infant was not able to maintain the level of his blood vitamin, whereas a second infant did maintain the level of cocarboxylase in his blood when given an intake ranging from 49 to 58 units of thiamin per day over the same period. This second infant, however, was the best infant we have ever studied so far as utilization of nutrients is concerned, and it is probable that the minimum requirement for the average infant is above the level of 60 units of thiamin per day which these infants received. These blood data tend to substantiate the conclusion based on urinary excretion that 80 units of intake represent the minimum requirement. Further investigations are now under way to determine the lowest thiamin intake which is compatible with health, and also the optimal amounts of vitamin B<sub>1</sub> for the best development of the infant.

11827 P

### Buccal Absorption of $\alpha$ -Estradiol in Propylene Glycol

UDALL J SALMON AND SAMUEL H GEIST

*From the Mt Sinai Hospital, New York*

In the treatment of estrogen deficiency states, it is frequently necessary to administer estrogens for prolonged periods of time

glycol<sup>1-6</sup> The published reports, to date, agree as to the absence of toxic effects unless administered in large doses

*Discussion* A simple and economical method of administering estrogens for therapeutic purposes is described This method consists of instilling several drops of a solution of  $\alpha$ -estradiol in propylene glycol in the sublingual space Definite morphologic evidence of absorption (demonstrated by characteristic estrogenic effects in the histologic sections of the vaginal mucosa and vaginal smears) was noted in all cases at the end of one week with daily doses of 0.2 and 0.3 mg of  $\alpha$ -estradiol

Although experimental studies appear to indicate that, in the small doses used, propylene glycol has no toxic effects further studies should be conducted in order to determine what the effect of continued administration of these small doses would be Should propylene glycol be found to be completely innocuous, this method of administration of estrogens offers great promise of simplifying and reducing the cost of estrogen therapy

11828

### Rumen Synthesis of the Vitamin B Complex<sup>\*†</sup>

M. I. WEGNER, A. N. BOOTH, C. A. ELVEHJEM AND E. B. HART

*From the Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison*

Work reported by McElroy and Goss<sup>1</sup> indicates the ability of sheep to synthesize several members of the vitamin B complex in

<sup>1</sup> Weatherby, J. H., and Haug, H. B., *J. Am. Pharm. Assoc.* 1938, **27**, 466

- Laug, E. P., Chervy, H. O., Morris, H. J., and Woodard, G., *J. Indust. Hyg. and Toxicol.* 1939, **21**, 173

<sup>2</sup> Hanzlik, P. J., Newman, H. W., Van Winkle, W., Lehman, A. J., and Kennedy, N. K., *J. Pharm. and Exp. Ther.* 1939, **67**, 101

<sup>3</sup> Seidenfeld, M. A., and Hanzlik, P. J., *J. Pharm. and Exp. Ther.* 1932, **44**, 109

Hanzlik, P. J., Lehman, A. J., Van Winkle, W., and Kennedy, N. K., *J. Pharm. and Exp. Ther.* 1939, **67**, 114

<sup>4</sup> Jansen, A. R., and Molitor, H., *J. Pharm. and Exp. Ther.* 1939, **67**, 89

<sup>\*</sup> Published with the approval of the Director of the Wisconsin Agricultural Experiment Station

<sup>†</sup> Thanks are extended to LaVell Henderson and Dr. Harry Weisman for assistance in the pyridoxine and nicotinic acid assays; to George Bahler for biotin determinations; and to Ann Earle and Bessie Zeman for pantothenic acid and riboflavin assays

<sup>1</sup> McElroy, L. W., and Goss, H., *J. Biol. Chem.* 1939, **130**, 439

objective evidence (demonstrated by vaginal smears† and vaginal biopsies) of estrogen deficiency. The hormone was administered 3 to 6 times a day. Vaginal smears were taken daily and vaginal biopsies at intervals of 1 to 2 weeks.

**Results** Characteristic vaginal smear changes indicating an estrogen effect were noted as early as 4 days after the beginning of hormone administration, with daily doses of 0.3 mg of  $\alpha$ -estradiol (Figs 1 and 2). Vaginal biopsies at the end of one week showed definite evidence of estrogen stimulation. No untoward effects were noted as a result of the propylene glycol absorption.

A number of experimental studies in animals and humans have been performed with regard to the possible toxicity of propylene

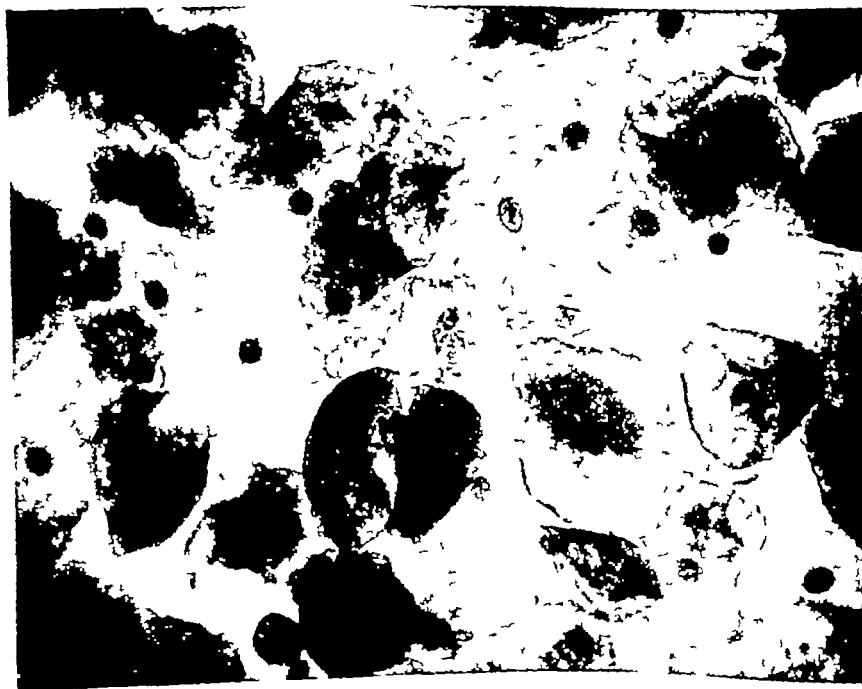


FIG 2

Vaginal smear taken 4 days after beginning of sublingual administration of  $\alpha$ -estradiol in propylene glycol. Dose: 0.5 mg  $\alpha$ -estradiol 6 times daily for 4 days = total of 1.2 mg = 14400 r u. Smear reveals typical estrogenic effect.

†Vaginal smears prepared by the aqueous fuchsin technique.<sup>8</sup>

<sup>7</sup>Salmon, U. J., and Frank, R. T., *Proc Soc Exp Biol and Med* 1936, **33**, 612.

<sup>8</sup>Geist, S. H., and Salmon, U. J., *Am J Obs and Gyn*, 1939, **38**, 392.

TABLE I.  
Assay Results  
( $\gamma$ /g dry matter)

Factor	Basal ration	Rumen contents	Rumen contents (vitamin B <sub>1</sub> added to ration)
Thiamin	0	10 12	> 20
Riboflavin	< 0 4	18 6	26 5
Nicotinic acid	60	220	172
Pantothenic acid	< 3 4	55 5	82 5
Pyridoxine	0	7	11 12
Biotin	< 0 018	0 087	0 250

In a recent report by Knight, *et al.*,<sup>9</sup> the rapid destruction of ingested vitamin C in the rumen was demonstrated. The possibility existed that the same destruction might occur with members of the B complex. To investigate this possibility 200 mg of thiamin were added to the calf ration (given above) and the rumen contents again assayed for the members of the B complex as listed. The results are recorded in the last column of Table I. Since the samples were taken 6 hours after feeding and the thiamin content was still appreciably higher than found in the previous experiment destruction of this factor in the rumen, if any, is contraindicated. Further, the addition of thiamin to the ration apparently stimulated the synthesis of the other members of the B complex except nicotinic acid as can be readily seen by comparing the two experiments, with and without added thiamin respectively.

The results were obtained through the use of reliable methods with a possible exception of nicotinic acid and even here the differences between the basal ration and rumen content are significant.

The synthesis of the vitamin B complex is very likely due to bacterial action in view of the stimulating effect obtained by adding thiamin to the ration fed.

*Summary* The ability of the bovine species to synthesize significant amounts of thiamin, riboflavin, nicotinic acid, pyridoxine, pantothenic acid and biotin when fed a ration very low in these compounds has been demonstrated. A destruction of thiamin in the rumen is contraindicated. At the same time there was an apparent stimulation of the synthesis of the other factors assayed for when thiamin was added to the "synthetic" ration.

<sup>9</sup> Knight, C. A., Dutcher, R. A., Guerrant, N. B., and Bechdel, S. I., *Proc. Soc. Exp. Biol. and Med.*, 1940, 44, 20.

the rumen. Significant increases in the vitamin content of rumen ingesta were found in comparison to the amounts in the ration fed. The vitamins determined were thiamin, riboflavin, pyridoxine, and pantothenic acid. The ration used consisted of washed casein 5, washed sardine meal 67, cerelese 110, corn starch 106, mineral mixture 30, and dried plain beet pulp 63.7%. In a later report<sup>2</sup> McElroy and Goss obtained similar results when feeding this ration to a cow with a rumen fistula.

At the time the above work was being reported a similar experiment was under way at this station. The animal used was a heifer calf with a rumen fistula. The ration fed consisted of acid-washed casein 4, urea 10, cod liver oil 10, salt mixture 30, corn molasses 100, corn starch 710, and bleached wood-pulp 100%. The calf was fed daily at 8 A M and 8 P M, receiving 2 pounds of ration per feeding. After the calf had received this ration for several weeks the rumen contents were sampled on alternate days at 2 P M until about 20 kilos of wet material had been obtained. The size of the samples was approximately 2 kilos of wet material (12-15% dry matter). Immediately upon sampling, the material was diluted with 95% ethyl alcohol to a final concentration of 45-50% and placed in a cold room (35°F) in order to stop bacterial action. Two to 3 days later this material was placed in enamel-lined pans and dried in a drying room for 24 to 40 hours at a temperature of 45 to 50°C. After grinding these samples were again stored in the cold room and removed as needed. The analyses were assays on a composite sample.

The components of the vitamin B complex assayed for were thiamin, riboflavin, nicotinic acid, pyridoxine, pantothenic acid, and biotin. The methods of assay used were as follows: Thiamin, chick,<sup>3</sup> riboflavin, microbiological,<sup>4</sup> nicotinic acid, chemical,<sup>5</sup> pyridoxine, rat,<sup>6</sup> pantothenic acid, microbiological,<sup>7</sup> biotin, microbiological.<sup>8</sup> The results are given in Table I.

From Table I it is readily seen that an appreciable synthesis of all members of the B complex assayed for occurs.

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<sup>2</sup> McElroy, L. W., and Goss, H., *J. Biol. Chem.*, 1940, **133**, lxxv.

<sup>3</sup> Arnold, A., and Elvehjem, C. A., *J. Nutr.*, 1938, **15**, 403.

<sup>4</sup> Snell, E. E., and Strong, F. M., *J. Ind. and Eng. Chem. Anal. Ed.*, 1939, **11**, 346.

<sup>5</sup> Melnick, D., and Field, H., Jr., *J. Biol. Chem.*, 1940, **134**, 1.

<sup>6</sup> Conger, T. W., et al., unpublished method not yet in press.

<sup>7</sup> Strong, F. M., et al., method unpublished. Reported to the Am. Chem. Soc., Div. Biol. Chem., 1940.

<sup>8</sup> Peterson, W. H., et al., method unpublished. Reported to the Am. Soc. Biol. Chemists, New Orleans, March 13-16, 1940. *J. Biol. Chem.*, 1940, **133**, lxxxv.

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<sup>9</sup> Knight, C. A., Dutcher, R. A., Guerrant, N. B., and Bechdel, S. I., *Proc. Soc. Exp. Biol. and Med.*, 1940, **44**, 90.



# Suitability of the Common Goldfish for Assay of Thyrotropic Hormone

AUBREY GORMAN (Introduced by J Frank Daniel)

*From the Department of Zoology and the Institute of Experimental Biology,  
University of California, Berkeley*

The unusual responsiveness of the thyroid tissue of goldfish to small amounts of hypophyseal thyrotropin has provided in these experiments the basis for a sensitive inexpensive assay procedure. In a series of tests in which 115 two-inch-long fish were used, marked growth of the epithelium of the thyroid follicles was produced by crude and relatively purified preparations from the pituitaries of mammals, birds, amphibia, and fishes. One Parkes-Rowlands guinea pig unit, 0.25 mg of a purified standardized mammalian pituitary thyrotropic powder was much larger than the minimum dose producing thyroid stimulation in the goldfish.

Groups of 5 fish receiving the same treatment were kept in one-gallon jars. The hormone was administered intraperitoneally in 5 successive daily injections of 0.2 cc each day dissolved or suspended in the desired dilution of Ringer's solution. At autopsy, performed on the sixth day, the lower jaw was removed and trimmed. This piece of tissue, about 2 mm by 3 mm in size, was fixed in Bouin's fluid which contains enough acid to eliminate the need of special decalcifying agents. The jaw was cut sagittally in 10 $\mu$  sections. Only about 100 serial-sections, estimated to be approximately median sagittal and parasagittal were saved. The height of the epithelium was measured with a calibrated ocular micrometer and an average of 10 measurements from different follicles was taken.

TABLE I  
Dose of Thyrotropic Preparation Required for Specified Response, Expressed as  
Milligrams of Desiccated Tissue \*

Reaction height epithelium, $\mu$	Pituitary preparations					
	Fish (sole)	Leopard frog	Bull frog	Chicken	Sheep	Parkes Th 9
20 $\mu$ 35 $\mu$	10.0	5.0	†	5.0	†	†
35 $\mu$ 50 $\mu$	†	10.0	5.0	10.0	†	†
50 $\mu$ 90 $\mu$	†	†		10.20	150	0.25—
90 $\mu$ †	†	†	20.0	†	†	5.0

\*Excepting the Parkes thyrotropic extract for which the dose is expressed as milligrams of purified powder.

†Lowest dosage was too high to produce this response.

‡Highest dosage was too low to produce this response.

The thyroid epithelial response in this cold-blooded animal was very uniform to any given dosage level. The average control epithelial cell measured  $1\mu$  in height, with extreme variations of  $0.5\mu$  from this dimension. In strongly stimulated thyroid tissue, epithelia as high as  $12\mu$  were seen. It was found convenient to recognize different grades of stimulation. A 50% increase in average follicle cell height (cells  $2\mu$  high) was considered the minimal reaction. Increases of 175% ( $3.5\mu$ ), 300% ( $5\mu$ ) and 500% (9 to  $10\mu$ ), respectively, were adjudged the arbitrary limits for delineation of the reactions referred to as mild, strong, and very strong.

## 11830

## Further Experiments on Nutritional Achromotrichia in Rats and Mice

PAUL GYÖRGY AND C. E. POLING\*

*From the Babies and Childrens Hospital, and the Department of Pediatrics, School of Medicine, Western Reserve University, Cleveland*

From the results of previous experiments it has been concluded that concentrates of pantothenic acid with a purification up to 40 to 50% appear to contain one factor but not the only factor concerned in the cure of nutritional achromotrichia in rats.<sup>1</sup>

Later it became evident that this factor, which proved to be heat labile in alkaline solution is identical with pantothenic acid.<sup>2</sup> In a group of rats kept on a diet free from pantothenic acid administration of daily doses of from 75 to 100  $\mu$ g of synthetic pantothenic acid† brought about cure of the nutritional achromotrichia in from 5 to 7 weeks. In some rats the cure was slower and in a few it was never quite complete.

These experiments were repeated with black mice‡ kept under

\* S. M. A. Corporation Fellow in Biochemistry assigned to the Department of Pediatrics.

1 György, P., Poling, C. E., and Subbarow, Y., *J. Biol. Chem.* 1940, 132, 759.

2 György, P., and Poling, C. E., *Science* 1940, 92, 202.

† Synthetic pantothenic acid was generously put at our disposal by Merck & Co. Inc., Rahway, N. J.

Grateful acknowledgment is made to Dr. Agnes Fay Morgan, University of California, Berkeley, for the strain of black rats used in the experiments on nutritional achromotrichia and to Dr. William C. Barrett, Department of Anatomy, School of Medicine of Western Reserve University, Cleveland, for the C 57 strain of black mice.

similar nutritional conditions. The diet used was particularly conducive<sup>3</sup> to the production of acrodynia in rats. It consisted of 18 parts of purified casein, § 10 of dried heated egg white, 58 of sucrose, 8 of melted butter fat, 2 of cod liver oil and 4 of salt mixture. Raw egg white was heated over a steam bath for 3 hours in order to destroy the injurious effect ("egg white injury"<sup>4</sup>) it has on animals. The diet fed both to the rats and the mice was supplemented with 20 µg each of thiamin chloride, pyridoxine and riboflavin.

In from 3 to 5 weeks, apart from the skin lesions, the fur of the mice became gray or brownish, similar in color to the fur of wild house mice or more silvery. Daily doses of from 50 to 100 µg of calcium pantothenate (synthetic Merck) produced quick and definite effect on the depigmentation of the fur, with practical cure in from 3 to 5 weeks. The therapeutic effect on the cutaneous manifestations was even more rapid.<sup>5</sup> These lesions were similar to those described in rats as Type II<sup>6</sup> and to those recently observed in mice under similar conditions by Norris and Hauschildt.<sup>7</sup>

A group of 24 black rats and 8 black mice were kept under prolonged observation without the institution of any further change in the experimental conditions. They received the supplement of pantothenic acid for as long as 6 months after the initial achromotrichia had been cured.

It became evident that the diet fed these animals could not be regarded as complete. Changes in the pigment metabolism of the fur again became apparent in both the rats and the mice. In addition there were (1) cutaneous lesions in some of the rats similar to those reported by Morgan and Simms,<sup>8</sup> (2) a predominantly scaly dermatosis and dandruff in some of the rats and mice and (3) a thinning of the pelt in a few rats and mice. The pathological changes in the content of pigment in the fur never reached the stage of depigmentation seen in rats and mice kept on a diet deficient in pantothenic acid, and they were characterized as a rule either by brownish discoloration of the black fur or by single gray hairs interspersed in the fur without generalized graying or by both. The brownish discoloration was more conspicuous than the moderate diffuse gray-

<sup>3</sup> György, P., and Eckardt, R. E., *Biochem J.*, 1940, **34**, 1143

§ Generously furnished by the S M A Corporation Chagrin Falls, Ohio

<sup>4</sup> György, P., *J Biol Chem.*, 1939, **131**, 733. György, P., Kuhn, R., and Lederer, E., *J Biol Chem.*, 1930, **131**, 745. Birch, T. W., and György, P., *J Biol Chem.*, 1939, **131**, 761

<sup>5</sup> György, P., and Poling, C. E., data to be published

<sup>6</sup> György, P., and Eckardt, R. E., *Nature (London)* 1939, **144**, 512

<sup>7</sup> Norris, E. R., and Hauschildt, J., *Science*, 1940, **92**, 316

<sup>8</sup> Norris, E. R., and Hauschildt, J., *J Nutrition* 1940, **10**, 233

ing, the fur in the latter case being only slightly different from normal fur. These secondary changes appeared usually from 4 to 12 weeks after the initial nutritional achromotrichia had been cured by the administration of pantothenic acid.

When 100  $\mu$ g of a highly purified concentrate of biotin<sup>9</sup> were added to the daily diet fed this group of rats and mice, or 0.25  $\mu$ g of crystalline biotin methyl ester<sup>11</sup> to the diet of 2 rats, definite and quick improvement in the pigment metabolism of the fur was noted.

These observations are in agreement with those made in rats kept on a diet that produced typical egg white injury,<sup>10</sup> the pelts of which showed distinct signs of depigmentation, such as mild graying, brownish discoloration and rusting. The present group of rats and mice was fed a diet which contained only a small amount of egg white (10%) and even this amount was detoxified to a large extent by heating. However, the possibility that it may have affected the pigment metabolism of the pelt cannot be denied. Be that as it may, the experiments here reported prove that there is a possible interrelationship between biotin and pigment metabolism of the fur in rats and mice.

The order in which pantothenic acid and biotin were administered was found to be of decisive importance in the present series of experiments. When supplements of biotin were given before supplements of pantothenic acid, achromotrichia was somewhat aggravated, and the curative effect of biotin became evident only when it was given during the period of secondary changes in the fur after the initial achromotrichia had been relieved by pantothenic acid.

In black rats and mice treated with pantothenic acid and biotin, pigmentation of the fur still did not necessarily become entirely normal. It should be borne in mind, however, that the fur of black rats kept on the stock diet<sup>11</sup> shows, as a rule, with progressing age, a slight brownish discoloration and is often interspersed with single gray hairs. The question arises whether this condition should be considered a function of age rather than one of incomplete diet.

**Conclusion.** Pantothenic acid is only one, although probably the most important factor in the prevention of nutritional achromotrichia, as it is seen in rats and mice used in experiments on the

<sup>9</sup> du Vigneaud, V., Melville, D. B., Grögg, P., and Rose, C. S. *Science* 1940, 92, 62.

<sup>10</sup> Kindly furnished by Professor F. Kögl, Utrecht, Netherlands.

<sup>11</sup> Grögg, P., in Pfandner, A., and Schlossmann, M., *Handbuch der Kinderheilkunde* 1935, 10, 55.

<sup>12</sup> Sherman, H. C., and Muhlfeld, M. *J. Biol. Chem.* 1922, 53, 41. Smith, A. H., and Bing, F. C. *J. Nutrition* 1925, 1, 179.

vitamin B<sub>2</sub> complex. Biotin is an additional factor that is connected with the maintenance of the normal pigment metabolism of the fur in rats and mice under special dietary conditions

## 11831

### Effects of Epinephrine and Amphetamine on Respiration and Blood Pressure in Different Postures

ROLLAND J MAIN

*From the Department of Physiology, Medical College of Virginia, Richmond*

The cause of the overventilation of the erect posture, as indicated by lowering of the alveolar CO<sub>2</sub> tension, is unknown. Main<sup>1</sup> suggested that it might be due to the fact that the pressure in the carotid sinus is lowered about 20 mm Hg on standing, which should therefore stimulate respiration. Turner<sup>2</sup> believed it might be due to cerebral ischemia. I decided to test the former hypothesis by raising the blood pressure of a standing subject, to see if this would remove the excess respiratory stimulation by increasing the pressure in the carotid sinus back to the normal level.

I decided to use epinephrine subcutaneously to raise the blood pressure. Two reports in the literature on the effect of epinephrine on alveolar CO<sub>2</sub> are somewhat at variance, Arnoldi<sup>4</sup> reporting a rise and Peters,<sup>5</sup> a slight fall. However, the side effects of this drug, such as marked tremor, cold perspiration, feeling of apprehension, and the possible production of a lactic acidemia,<sup>6</sup> made it advisable to control the results with some other vasopressor drug which would not produce such deleterious side effects. Amphetamine sulfate subcutaneously was selected because of its entirely different side effects—euphoria and the complete lack of tremor and sweating.

It was soon found that the subjects, males between the ages of 20 and 30, varied greatly in their response to epinephrine—some being severely affected by 0.5 cc, and others showing very little ill effects from 0.75 cc of a 1 to 1000 solution in ampules (Parke-Davis). No ill effects were noticed in any case from amphetamine

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<sup>1</sup> Main, R, *Va Med Monthly*, 1937, **64**, 330

<sup>2</sup> Loman, J, and Myerson, A, *Am J Psychiat*, 1936, **92**, 791

<sup>3</sup> Turner, A, *Am J Physiol*, 1927, **80**, 601

<sup>4</sup> Arnoldi, W, *Deut Med Woch*, 1924, **50**, 1397

<sup>5</sup> Peters, J, *Am J Physiol*, 1917, **44**, 84

<sup>6</sup> Cori, C, *J Biol Chem*, 1925, **63**, 253

sulfate in doses up to 30 mg. Care was always taken not to make an accidental intravenous injection and not to massage the area after injection. Not more than one injection a week was given to a subject, in order to prevent any possible adaptation. A total of 12 subjects was used, in 12 experiments with amphetamine, and 24 with epinephrine.

After eliminating a few subjects who were very sensitive to the effects of epinephrine, I found it necessary to administer from 0.5 to 0.75 cc of a 1 to 1000 solution, in order to obtain a maximal increase in systolic blood pressure of about 20 mm Hg. From 20 to 30 mg of amphetamine sulfate subcutaneously was required to produce a similar effect.

The subjects came to the laboratory within an hour after having eaten, so that hunger contractions would not intervene toward the end of the experiment to disturb the  $\text{CO}_2$  tension. They were not permitted to have coffee at the previous meal or to smoke one hour beforehand. They would lie down for at least 30 minutes before the experiment was started. The effect of the drug was tested in the supine position the entire period or at other times was given while standing.

Alveolar  $\text{CO}_2$  samples were usually taken every 10 minutes to cover normal variations in tension and unconscious errors in obtaining the sample. The Haldane-Priestley method was used, the forced expiration being made at the end of a normal expiration.

Fig. 1 illustrates the typical effect of epinephrine (0.6 cc subcutaneously) on the same subject lying and standing. With the doses of epinephrine used throughout (0.25 to 0.75 cc) there was never noted any distinct effect upon alveolar  $\text{CO}_2$  tension even when the blood pressure of the standing subject rose over 20 mm Hg. Apparently any possible lactacidemia produced by these doses of epinephrine is inadequate to affect the acid-base balance of the blood. Neither the distinct tremor nor the apprehension produced by the drug seemed to have any effect upon the  $\text{CO}_2$  tension.

The effect of amphetamine sulfate was tested in the same way as with epinephrine. There was always a distinct feeling of euphoria which lasted less than 2 hours and had disappeared by the end of the experiment. No effect on alveolar  $\text{CO}_2$  was noticed with this drug even when it caused a distinct increase in blood pressure.

I would estimate that from its maximum effect on blood pressure (but not duration) that 20 to 25 mg of amphetamine sulfate is equivalent to about 0.6 cc of 1:1000 epinephrine by subcutaneous administration.

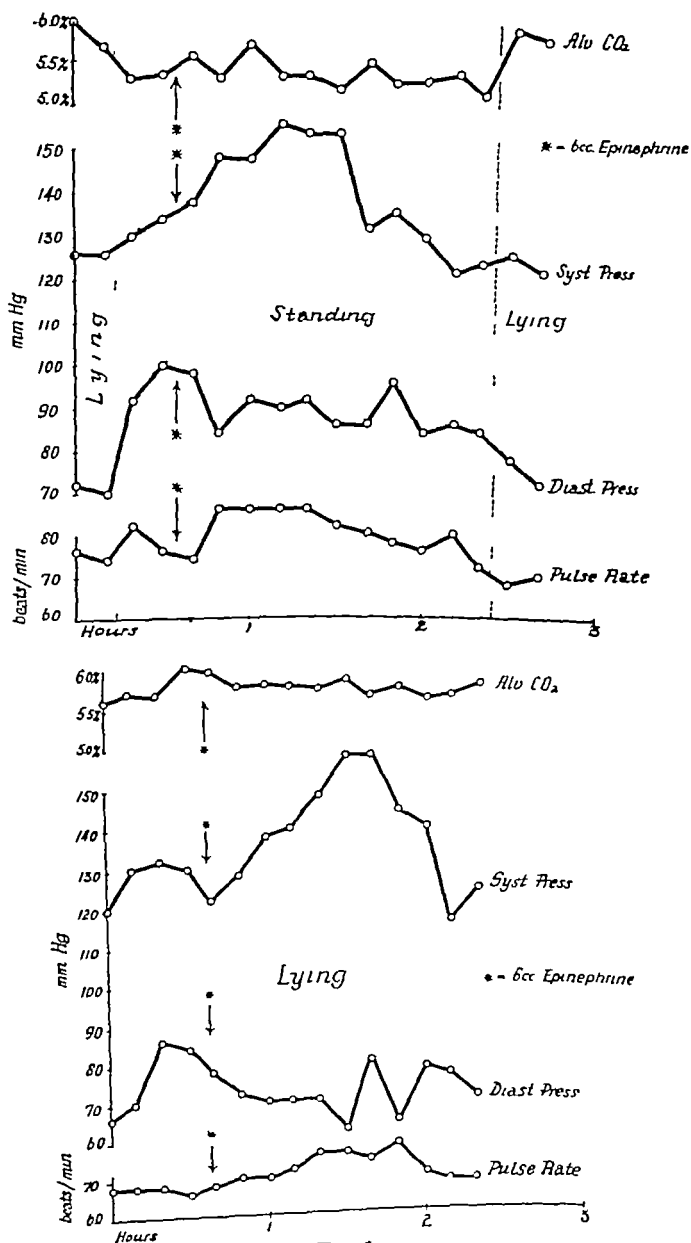


FIG 1.

Effects of 0.6 cc epinephrine (1:1000) subcutaneously on the same subject in different postures

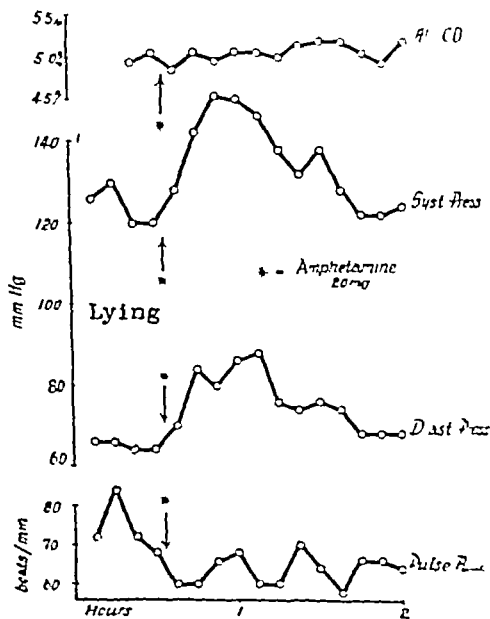
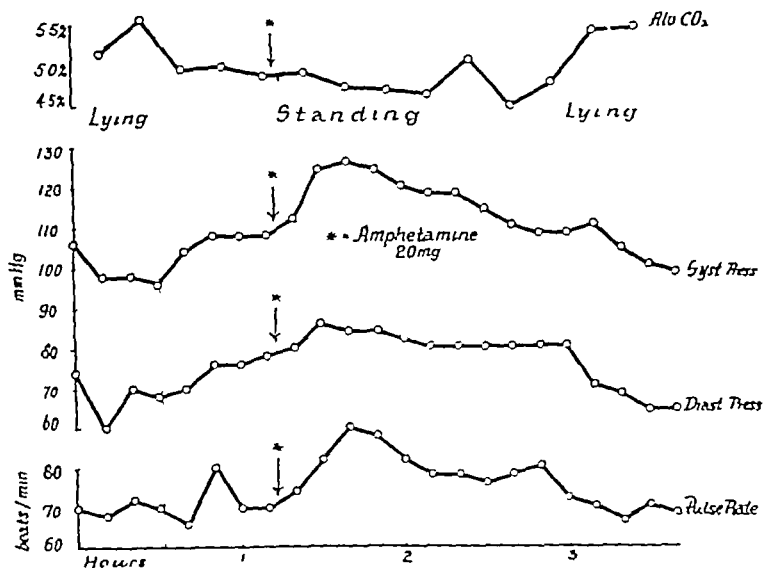


FIG 2

Effects of 20 mg amphetamine sulfate subcutaneously to one subject in different postures

Fig 2 illustrates the effect of 20 mg of amphetamine sulfate on the same subject lying and standing. Considerable variation was



found, even in the same individual from day to day. A dose that would produce a rise of 20 mm Hg in blood pressure in one day, might produce a rise of only half as much the next week. Neither drug had a distinct effect upon the respiratory rate.

Since increasing the systemic blood pressure and thereby probably restoring the normal pressure in the carotid sinus, did not restore the alveolar  $\text{CO}_2$  to normal, we must assume that the overventilation of standing may not be due to the drop in blood pressure in the carotid sinus and we are forced back to the remaining hypothesis, that the respiratory stimulation is due to a relative ischemia of the brain. These results would indicate that the rise in alveolar  $\text{CO}_2$  on bending over while standing, may be due not to the return to normal pressure in the carotid sinus, but rather to relief of the cerebral ischemia. It at first seemed illogical that the drop in blood pressure in the carotid sinus on standing should not cause respiratory stimulation, but it may be that the probable increase of pressure in the aorta on standing exactly neutralizes the drop in blood pressure in the carotid sinus.

*Conclusion* Neither epinephrine nor amphetamine in the doses used affected the alveolar  $\text{CO}_2$  tension of subjects when standing or lying. Consequently the lowered alveolar  $\text{CO}_2$  of the erect posture may be due to a relative cerebral ischemia.

This work was made possible by a grant from the Council on Pharmacy and Chemistry of the American Medical Association.

11832

### Twenty-four Hour Response to Androgens in the Immature Male Rat \*

R. R. GREENE AND M. W. BURRILL. (Introduced by A. C. Ivy.)

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Astwood<sup>1</sup> has demonstrated that estrogens cause a measurable increase in the uterine weight of the immature rat within 6 hours after administration. The weight increment which is largely due to increased hydration of the uterine tissues forms the basis for his

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\* Aided by a grant from Ciba Pharmaceutical Products, Inc.

<sup>1</sup> Astwood, E. B., *Endocrinology*, 1938, **23**, 25

estrogen assay method Astwood later stated<sup>2</sup> that androgens have the same effect on the rat uterus. This latter finding has been confirmed.<sup>3</sup> The present investigation was undertaken to determine whether or not androgens have a similar weight-increasing effect on the sexual accessories of the immature male rat.

One hundred and thirty-one male rats, 32 to 38 days old and 65 to 85 g in weight were used. Twenty-one animals served as controls. The rest were divided into 5 groups which were given various doses of testosterone propionate† in 0.1 cc of peanut oil in a single injection subcutaneously. Twenty-four hours after treatment the animals were killed. The ventral prostates and seminal vesicles were carefully removed under a dissecting microscope and immediately weighed on a Roller-Smith micro-balance. The dosages used and the average weights of the removed organs are presented in tabular form (Table I).

With the exception of the lowest dosage group (0.00625 mg) the prostates showed a uniform and progressive increase in mean weights with increasing dose. The seminal vesicles showed a relatively greater response to androgens in the 24-hour period. The increase in mean seminal vesicle weight was consistent with the increased dose given.

The mean weights of both prostates and seminal vesicles obtained with 0.5 mg testosterone propionate were significantly greater than those of the controls (critical ratios of 3.36 for the prostates and 4.63 for the seminal vesicles). The mean seminal vesicle weight with 0.1 mg testosterone propionate was also significantly greater than that of the control (critical ratio 3.64) but the mean prostate weight at this dose was not. The differences between the lower dosage groups and the control group were not significant.

TABLE I

Test prop mg	No animals	M body wt	M prostate wt		C <sub>c</sub> increase over cont	M seminal ves wt		C <sub>c</sub> increase over cont
			mg	σ		mg	σ	
—	21	75.0	45.90	2.38	—	12.08	0.87	—
0.00625	25	74.0	42.62	1.80	—7.14	13.36	0.62	10.59
0.025	20	73.9	47.85	2.22	4.24	14.43	1.06	19.46
0.1	25	73.8	48.23	2.12	5.07	15.15	0.87	25.41
0.10	20	73.5	51.53	2.01	12.26	16.49	0.83	31.51
0.5	20	74.5	59.05	2.38	*30.61	18.54	1.08	53.47

σ = Standard deviation of the mean

= Significant difference from control mean

<sup>2</sup> Astwood E. B. *Proc. Am. Physiol. Soc.* 1940, p. 6

<sup>3</sup> Greene R. R. and Harris S. C. *Proc. Soc. Exp. Biol. and Med.* 1940 45: 24

† Testosterone propionate (Perandren) furnished by courtesy of Ciba Pharmaceutical Products, Inc.

found, even in the same individual from day to day. A dose that would produce a rise of 20 mm Hg in blood pressure in one day, might produce a rise of only half as much the next week. Neither drug had a distinct effect upon the respiratory rate.

Since increasing the systemic blood pressure and thereby probably restoring the normal pressure in the carotid sinus, did not restore the alveolar  $\text{CO}_2$  to normal, we must assume that the overventilation of standing may not be due to the drop in blood pressure in the carotid sinus and we are forced back to the remaining hypothesis, that the respiratory stimulation is due to a relative ischemia of the brain. These results would indicate that the rise in alveolar  $\text{CO}_2$  on bending over while standing, may be due not to the return to normal pressure in the carotid sinus, but rather to relief of the cerebral ischemia. It at first seemed illogical that the drop in blood pressure in the carotid sinus on standing should not cause respiratory stimulation, but it may be that the probable increase of pressure in the aorta on standing exactly neutralizes the drop in blood pressure in the carotid sinus.

*Conclusion* Neither epinephrine nor amphetamine in the doses used affected the alveolar  $\text{CO}_2$  tension of subjects when standing or lying. Consequently the lowered alveolar  $\text{CO}_2$  of the erect posture may be due to a relative cerebral ischemia.

This work was made possible by a grant from the Council on Pharmacy and Chemistry of the American Medical Association.

## 11832

### Twenty-four Hour Response to Androgens in the Immature Male Rat \*

R. R. GREENE AND M. W. BURRILL (Introduced by A. C. Ivy)

*From the Department of Physiology and Pharmacology, Northwestern University Medical School, Chicago*

Astwood<sup>1</sup> has demonstrated that estrogens cause a measurable increase in the uterine weight of the immature rat within 6 hours after administration. The weight increment which is largely due to increased hydration of the uterine tissues forms the basis for his

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\* Aided by a grant from Ciba Pharmaceutical Products, Inc.

<sup>1</sup> Astwood, E. B., *Endocrinology*, 1938, **23**, 25

estrogen assay method Astwood later stated<sup>2</sup> that androgens have the same effect on the rat uterus This latter finding has been confirmed<sup>3</sup> The present investigation was undertaken to determine whether or not androgens have a similar weight-increasing effect on the sexual accessories of the immature male rat

One hundred and thirty-one male rats, 32 to 38 days old and 65 to 85 g in weight were used Twenty-one animals served as controls The rest were divided into 5 groups which were given various doses of testosterone propionate† in 0.1 cc of peanut oil in a single injection subcutaneously Twenty-four hours after treatment the animals were killed The ventral prostates and seminal vesicles were carefully removed under a dissecting microscope and immediately weighed on a Roller-Smith micro-balance The dosages used and the average weights of the removed organs are presented in tabular form (Table I)

With the exception of the lowest dosage group (0.00625 mg) the prostates showed a uniform and progressive increase in mean weights with increasing dose The seminal vesicles showed a relatively greater response to androgens in the 24-hour period The increase in mean seminal vesicle weight was consistent with the increased dose given

The mean weights of both prostates and seminal vesicles obtained with 0.5 mg testosterone propionate were significantly greater than those of the controls (critical ratios of 3.36 for the prostates and 4.63 for the seminal vesicles) The mean seminal vesicle weight with 0.1 mg testosterone propionate was also significantly greater than that of the control (critical ratio 3.64) but the mean prostate weight at this dose was not The differences between the lower dosage groups and the control group were not significant

TABLE I

Test prop mg	No animals	M body wt	M prost wt		% increase over cont	M sem ves wt		% increase over cont
			mg	$\sigma$		mg	$\sigma$	
—	21	75.0	45.90	2.38	—	12.08	0.87	—
0.00625	25	74.0	42.62	1.80	-7.14	13.36	0.62	10.59
0.025	20	73.9	47.85	2.22	4.24	14.43	1.06	19.46
0.1	25	73.8	48.23	2.12	5.07	15.15	0.87	25.41
0.10	20	73.5	51.53	2.01	12.26	*16.49	0.83	36.51
0.5	20	74.5	59.95	2.35	*30.61	*18.54	1.08	53.47

$\sigma$  = Standard deviation of the mean

\* = Significant difference from control mean

<sup>2</sup> Astwood F B *Proc Am Physiol Soc* 1940 p 6

<sup>3</sup> Greene R B and Harris S C *Endoc Soc Exp Biol and Med* 1940 45:24

† Testosterone propionate (Perandren) furnished by courtesy of Ciba Pharmaceuticals Products Inc

Within groups the individual prostate and seminal vesicle weights varied considerably. Since the animals used in this procedure also varied in age (32 to 38 days) and in weight (65 to 85 g) it seems probable that some of the intragroup variation in weight of the accessories may be due to these factors. In spite of the intragroup variation, however, the mean seminal vesicle weights in the treated groups showed a fairly consistent direct relationship to the logarithm of the dose as is characteristic of biological responses.

It is possible that a procedure similar to this might be useful as an assay method. The matter of greater accuracy (less group variability), the specificity of the response and the possibility of a shorter time period are being investigated.

*Summary* Twenty-four hours after the administration of testosterone propionate to the immature male rat there is an increase in the weights of the prostate and seminal vesicle. This weight increment is proportional to the dosage used.

### 11833 P

#### Infusion of Blood and Other Fluids into the Circulation via the Bone Marrow

L M TOCANTINS AND J F O'NEILL

*From Division of Hematology and Surgical Service B, the Jefferson Medical College and Hospital, Philadelphia*

Substances injected into the bone marrow enter the general circulation apparently unchanged and almost as rapidly as when injected intravenously<sup>1</sup>. In the parenteral administration of fluids to adult men it is sometimes impossible to use the intravenous route. Such is the case in widespread mutilations, burns, oedema, poorly developed, obliterated veins states of shock. In the newborn and in early infancy the venous system is so poorly developed that the superior longitudinal sinus reached through the anterior fontanelle is often the only available direct path to the circulation. The risk of injecting substances through this route is high. These facts seemed to justify an attempt at a wide clinical application in man of the intramedullary route for parenteral therapy. Trial of this route has been carried out in 14 persons. Ten infusions of citrated blood were given by this method to 7 patients, plasma infusions to 2 patients.

<sup>1</sup> Tocantins, L M, *Proc Soc Exp Biol and Med*, 1940, 45, 202

and infusions of 5% glucose and salt solution to 4 patients. In 17 trials there was one failure (Case 10), little marrow could be aspirated from the sternum and no fluid could be injected by gravity. In 2 children the fluids were introduced into the tibia and femur. In all the other patients the sternum or the clavicle were used. From 100 to 1050 cc have been injected, the infusion needle being left in place for a duration of from 1½ to 16 hours. Some signs of discomfort are evident during the first steps of the procedure (aspiration of marrow and injection of salt solution with a syringe). Throughout the course of the infusion practically no discomfort has been felt. The average rate of infusion varied between 0.4 and 9 cc per minute. Strict asepsis has been observed throughout. Indications that the infused citrated blood had been rapidly absorbed were a substantial increase in the amount of hemoglobin and number of erythrocytes in the blood of the patients 24 hours after the infusion. Readings of the intramedullary pressure as obtained with a water manometer before the start and after completion of the infusion have not differed by a significant margin. The readings varied between 50 and 120 mm of water and often corresponded closely with readings of the pressure in the veins of the forearm. There have been no local or constitutional reactions as evidenced by clinical or X-ray findings following any of the infusions. The 2 children that received the blood infusions were under one year of age and in neither of them were there veins available for transfusions. The method and apparatus used and the results obtained will be described in detail elsewhere.

Within the limitations outlined the intramedullary route for the parenteral administration of blood and other fluids seems to have its indications and has proved to be feasible. Application of the method should be limited to such times as when the intravenous route is not available and only by those familiar with the technique and favorable points of approach. Work now in progress indicates that other bones may have equal or greater advantages over those previously employed.

*Summary.* The intramedullary route for parenteral therapy has proved practicable in 16 out of 17 trials in 14 patients. Citrated blood, plasma, glucose and salt solutions have been infused without any immediate or delayed local or constitutional reactions.

Within groups the individual prostate and seminal vesicle weights varied considerably. Since the animals used in this procedure also varied in age (32 to 38 days) and in weight (65 to 85 g) it seems probable that some of the intragroup variation in weight of the accessories may be due to these factors. In spite of the intragroup variation, however, the mean seminal vesicle weights in the treated groups showed a fairly consistent direct relationship to the logarithm of the dose as is characteristic of biological responses.

It is possible that a procedure similar to this might be useful as an assay method. The matter of greater accuracy (less group variability), the specificity of the response and the possibility of a shorter time period are being investigated.

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<sup>1</sup> Tocantins, L. M., *Proc Soc Exp Biol and Med*, 1940, 45, 202

## Chromodacryorrhea and Parasympathetic Action of Cyclopropane

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The effects of anesthetic drugs upon the autonomic nervous system are of recent interest. Those of ether upon the sympathetic division have been described.<sup>1</sup> Parasympathetic stimulation by cyclopropane has been suggested from laboratory observations in dogs.<sup>2</sup> The results of a study of this latter action and its *modus operandi* are contained in this report. Cyclopropane does not inhibit the activity of cholinesterase of human serum *in vitro*.<sup>3</sup> Two of us<sup>4</sup> have noted that perfusion of turtles and frogs' hearts with solutions of cyclopropane produced parasympathetic effects. Perfusates from eserimized stomachs and hearts exhausted of the cyclopropane likewise produced parasympathetic stimulation when added to fresh eserimized hearts or to preparations of the frog's rectus abdominis muscle. Such results suggested the liberation of an acetylcholine-like substance. These results were not obtained with ethylene and ether. Recently Tashiro *et al.* described a technique for the assay of acetylcholine by the response of chromodacryorrhea or "red tears" in the eserimized albino rat. This procedure was utilized here to demonstrate further the parasympathetic action of cyclopropane.

**Procedure and Results.** Sixty-one observations were made on 36 white adult male rats weighing 225 to 325 g. Fifty gamma of eserine salicylate per 100 g of rat were given intraperitoneally. Twenty minutes later ether, cyclopropane, or ethylene narcosis was induced and maintained in the first plane of surgical anesthesia for a minimum of 20 minutes. The eyes were examined for the presence of chromodacryorrhea: (1) 20 minutes after the injection of eserine before anesthesia was begun, (2) immediately after anesthesia was discontinued and (3) 20 minutes after recovery. Thirty control observations were made on non-premedicated, unanesthetized rats.

<sup>1</sup> Bhattacharya, B. B., and Burn, J. H. *J. Physiol.* 1933, **78**, 257.

<sup>2</sup> Seivers, M. H., Meek, W. I., Rovenstine, E. A., and Stokes, J. A. *J. Pharm. and Exp. Therap.*, 1934, **51**, 1.

<sup>3</sup> Adriani, J., and Rovenstine, E. A., *Ines. and Anal.* 1940, in press.

<sup>4</sup> Adriani, J., and Rovenstine, E. A. *Am. J. Physiol.*, 1940, **120**, 299.

Tashiro, S., Smith, C., Badger, F., and Kozur, F. *Proc. Soc. Exp. Biol. and Med.* 1940, **44**, 678.



## 11834 P

## Tissue Ascorbic Acid in Hypophysectomized Rats

DONALD E. BOWMAN, LUCIEN E. MORRIS AND JOHN R. STACY  
(Introduced by Edward Muntwyler)

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Some time ago it was observed that the post-operative administration of crystalline ascorbic acid to hypophysectomized rats caused a very marked and sudden improvement in some cases in which recovery otherwise appeared quite doubtful. The tissue ascorbic acid was estimated in some of the untreated rats which failed to recover and it was found to be very low. Although the full significance of these preliminary observations is not clear a brief account may be of interest.

The 6-week-old rats were maintained since weaning entirely on Ralston Purina Dog Chow which was found to be scorbutogenic for guinea pigs. The ascorbic acid of the tissues was estimated in the usual manner by titrating a metaphosphoric acid extract of the tissues with 2,6-dichlorophenol indophenol. That the tissue ascorbic acid values in the 5 hypophysectomized rats examined are definitely below normal is indicated by the data of Table I. Some of the rats had received daily glucose injections and in all cases the stomachs contained food at autopsy. Hemorrhagic areas in various degrees of severity were also observed. The corresponding values for control non-hypophysectomized normal rats, determined at the same time, fell within the normal limits.

These findings indicate that a dietary intake or the injection of ascorbic acid after hypophysectomy in rats may prove highly beneficial. A more detailed study of the ascorbic acid metabolism of such animals is proposed.

TABLE I  
Tissue Ascorbic Acid Values in Hypophysectomized and Normal Rats

	mg per 100 g of tissue			
	Liver	Kidney	Adrenal	Small intestine
Avg 5 hypophysectomized rats	8.36	6.5	160.8	4.8
Max	10.25	8.2	164	9.6
Min	7.2	4.8	89.8	2.0
Avg for 5 normal rats	23.2	15.2	340	21.2
Max	30.1	19.1	450	26.4
Min	16	12.3	150	16.2

## Physical Allergy and "Auto-Antigens"

EDWARD H. RICHARDSON, JR. (Introduced by Arnold R. Rich)

*From the Department of Pathology, The Johns Hopkins Medical School,  
Baltimore, Md*

In an attempt to determine whether physical allergies could be explained on an immunological basis Karady,<sup>1</sup> in recent experiments, subjected a number of guinea pigs to heat and another group to cold in the manner described below. He found that a re-exposure to the same stimulus at the end of 2 weeks produced anaphylactic shock in the animals so treated, and in some of the guinea pigs the shock was severe enough to cause death. He also injected guinea pigs with guinea pig serum that had been subjected to heat and others with serum that had been subjected to cold. After 2 weeks the reinjection of heated or chilled serum into the animals that had previously been injected with heated or chilled serum respectively produced signs of anaphylaxis, and at autopsy the animals showed the pulmonary emphysema characteristic of anaphylactic shock. On the basis of these results Karady suggested that a physical stimulus, such as heat or cold, might so alter the protein of the blood plasma as to form an "auto-antigen" within the animal's own body, so that a re-exposure to the same stimulus would cause anaphylactic shock as in the above experiments. It was thought desirable to repeat these experiments and, if they could be confirmed, to study the mechanism of the sensitization. To this end we used the methods described by Karady, as follows:

Serum was obtained from normal guinea pigs, and half was heated at 56°C for 15 minutes and the other half cooled to -5°C for 15 minutes. Three cc of the heated serum was then injected into each of 7 guinea pigs by the intraabdominal route and 7 guinea pigs were injected with the same amount of cold-treated serum. When these injections were repeated at the end of 2 weeks the animals were observed closely for an hour following the injection and during that period no signs of anaphylaxis were seen in any of them. On the contrary their behavior seemed perfectly normal, and all survived.

In our next experiment, following the procedure of Karady, we subjected 12 male guinea pigs (which were somewhat larger than those used by Karady) to cold by immersing their hind limbs in an

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<sup>1</sup> Karady, S. *J Immunol*, 1939, 37, 457.

TABLE I  
Chromodacryorrhea in Eserinized Rats

Anesthetic agent	Premedication	Observations No	No of rats showing chromodacryorrhea		
			Before Anesthesia	At end of Anesthesia	Twenty minutes after recovery
None	Eserine	64	3	0	0
Cyclopropane	None	18	0	0	0
Ether	"	12	0	0	0
Cyclopropane	Eserine	23	0	17	1
Ethylene	"	18	0	0	1
Ether	"	20	0	0	2

and on those anesthetized with cyclopropane and ether without the administration of eserine. Twelve rats were treated with acetylcholine and the occurrence of chromodacryorrhea noted, thus confirming the studies of Tashiro, *et al*.<sup>5</sup>

A positive response for chromodacryorrhea was obtained in 18 of 23 eserinized rats treated with cyclopropane (Table I). This response was noted immediately after anesthesia was terminated. It was delayed in one rat which showed a positive response 20 minutes after recovery. The response was negative in the remaining 5 anesthetized with cyclopropane. Two of the 20 rats anesthetized with ether and one of the 18 treated with ethylene developed a positive response. Three unanesthetized eserinized rats similarly showed chromodacryorrhea. In none of the non-premedicated unanesthetized rats or the non-premedicated rats anesthetized with cyclopropane or ether was chromodacryorrhea observed.

**Discussion** Since chromodacryorrhea follows the injection of acetylcholine in eserinized rats,<sup>5</sup> the positive responses obtained from cyclopropane suggest that this drug liberates acetylcholine in rat tissue. The exceptional results obtained with the eserinized rats anesthetized with ether and ethylene may be due possibly to an increased acetylcholine secretion incident to handling or to variations in individual threshold since chromodacryorrhea may be elicited with larger doses of eserine. This may also account for the absences of chromodacryorrhea in the 5 eserinized rats given cyclopropane.

**Summary** The phenomenon of chromodacryorrhea produced by cyclopropane in eserinized white rats indicates that acetylcholine is liberated in mammalian tissues and supports the contention that the drug is a parasympathetic stimulant.

abdominal fibroids also were induced in guinea pigs injected subcutaneously with estrogens in the course of 4 months.<sup>2</sup> Fibroids appear even if quantities as small as 5% of the monobenzoic ester of estradiol per injection or a total of 400% are given in the course of 3 months.<sup>7</sup> All estrogens, free or esterified, natural or synthetic, proved to be tumorigenic.<sup>3</sup> Tablets of estradiol introduced beneath the skin may induce fibroids as early as in 3 weeks.<sup>4</sup> There is an enormous variability as to the localization of uterine and extra-uterine fibroids although when a sufficient number of animals is observed, some typical localizations can be established. A survey of these localizations of experimental fibroids is given in the present paper (Figs 1-16) based on the results of the autopsy of several hundred females to which the following 10 estrogens were administered: estrone, estrinol, estradiol, 4 esters of the latter (monobenzoate, monocaprylate, dipropionate, 17-benzoate-3-n-butyrate), stilbestrol, dipropionate of stilbestrol, hexestrol.

*Uterine tumors* are mostly subserous (Figs 3, 4, 14-C) and can become pedunculated (Fig 2). Very often they are to be found in the mesometrium (Figs 6, 8), intramural fibroids and those of the submucosa are rare (Fig 9). The whole surface of the uterine stem and part of the uterine horns may be covered by an extensive fibroid (Fig 1). The angle between the horns is often the site of a tumor. There may be also a chain of small tumors on the ventral surface of the uterus (Fig 5).

Subserous fibroids may be present also on the vagina and very rarely in the vaginal submucosa (Fig 10). In the *castrate* guinea pig an almost constant site of fibroids is near the upper ends of the tubes (Figs 4, 5); these apical tumors probably originate in the mesosalpinx and can attain an enormous size (Figs 7, 14-C) especially when joining with neighboring tumors of the spleen (Fig 4) or the abdominal wall. The apical tumor can descend also into the pelvis (Fig 14-C). Sometimes enormous tumoral masses en-

<sup>1</sup> Nelson, W. O., *Anat. Rec.* 1937, **68**, 99. *Endocrinology* 1939, **24**, 50. Mori-  
card, R., and Cruchot, J. *C. R. Soc. Biol. (Paris)* 1938, **129**, 556.

<sup>2</sup> Lipschütz, A., and Iglesias, R. *C. R. Soc. Biol. (Paris)* 1938, **129**, 519.  
Iglesias, R. *Public Med. Exp. (Chile)* 1938.

<sup>3</sup> Lately we have found that fibroids can be induced by as little as 2% per injection or a total of only 80% of estradiol in the course of 3 months when the monocaprylate ester is used (Lipschütz, Vargas, Baeza Rosales and Baeza Herrera, to be published soon).

<sup>4</sup> See Lipschütz, Vargas, Keref, Jedlicky, Belloho, Murillo, Rodríguez, Chaurum, Szabo, Ruiz. *C. R. Soc. Biol. (Paris)* 1938-1939, **129**, 131. *The Lancet* 1939-1940. *Public Med. Exp. (Chile)* 1939-1940. *Per. Chil. Hig. y Med. Prevent.* in press.

<sup>5</sup> Lipschütz, A., and Vargas, L. Jr. *The Lancet* 1939, **1**, 1313.

ice-salt mixture at  $-5^{\circ}\text{C}$  for 2 minutes. At the end of 2 weeks the immersion was repeated without any apparent effect on the animals which were carefully observed for an hour following the second exposure. All of them survived.

In order to be certain that the size of the animals had nothing to do with their lack of response to this treatment we repeated this portion of the experiment using 10 male guinea pigs all of which weighed between 200 and 250 g (the size of those used by Karady). Again the second exposure was entirely without effect in producing any signs of shock. After another lapse of 2 weeks their hind limbs were again immersed in the ice-salt mixture. Following this third exposure they were carefully observed for a period of one hour and once more failed to show any anaphylactic response. All animals survived.

Finally, another group of 7 male guinea pigs (weighing 200 to 250 g) were subjected to heat by immersing their hind limbs in water at  $56^{\circ}\text{C}$  for 2 minutes. Three weeks later they were again subjected to the same treatment, but no signs of anaphylaxis were observed in any of them during the hour following the re-exposure, and all survived.

*Summary.* We have been unable to confirm the report of Karady that guinea pigs can be sensitized anaphylactically to heat or cold by heating or chilling their limbs. We were also unable to confirm Karady's report that guinea pigs can be sensitized to heated or chilled guinea pig serum.

11837

### Uterine and Extrauterine Localizations of Experimental Fibroids Induced in Guinea Pig by Prolonged Administration of Estrogens\*

ALEXANDER LIPSCHUTZ, RIGOBERTO IGLESIAS AND LUIS VARGAS JUN. (Introduced by Emil Witschi)

*From the Department of Experimental Medicine, National Health Service of the Republic of Chile, Santiago*

The occurrence of uterine fibroids was reported in a number of guinea pigs treated for several months with estrogens<sup>1</sup>. *Extrauterine*

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\* This investigation has been aided by grants from The Jane Coffin Childs Memorial Fund for Medical Research, The Ella Sachs Plotz Foundation for the Advancement of Scientific Investigation, and Mr Adolfo Eastman of Lima (Chile). The esterified estrogens were generously supplied by Dr Carl Miescher of Messers Ciba, Basel, and the artificial ones by Professor Dodds, London.

abdominal fibroids also were induced in guinea pigs injected subcutaneously with estrogens in the course of 4 months<sup>2</sup> Fibroids appear even if quantities as small as 5% of the monobenzoic ester of estradiol per injection or a total of 400% are given in the course of 3 months<sup>†</sup> All estrogens, free or esterified, natural or synthetic, proved to be tumorigenic.<sup>3</sup> Tablets of estradiol introduced beneath the skin may induce fibroids as early as in 3 weeks<sup>4</sup> There is an enormous variability as to the localization of uterine and extra-uterine fibroids, although when a sufficient number of animals is observed, some typical localizations can be established A survey of these localizations of experimental fibroids is given in the present paper (Figs 1-16) based on the results of the autopsy of several hundred females to which the following 10 estrogens were administered estrone, estriol, estradiol, 4 esters of the latter (monobenzoate, monocaprylate, dipropionate, 17-benzoate-3-n-butyrate), stilbestrol, dipropionate of stilbestrol, hexestrol

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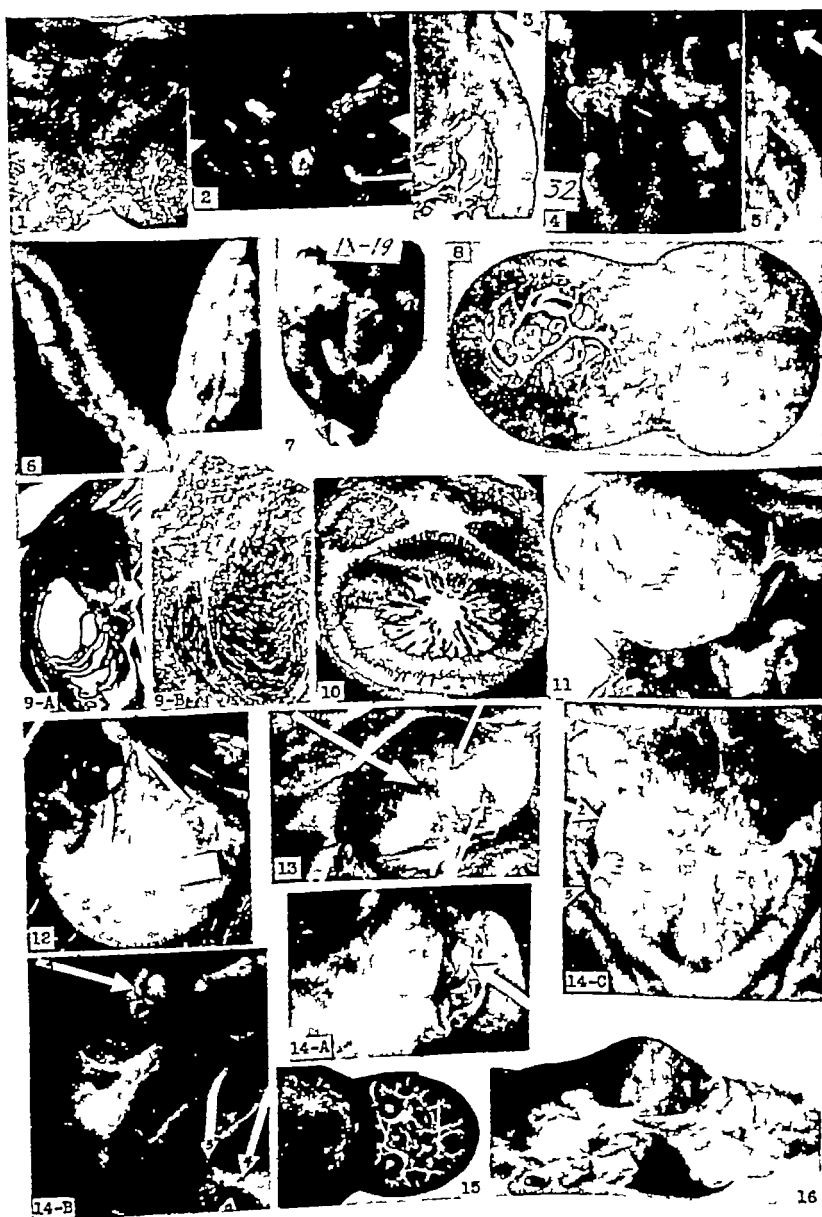
<sup>1</sup> Nelson, W O, *Anat Rec* 1937, **68**, 99, *Endocrinology*, 1939, **24**, 50, Moricard R., and Cauchoux, J, *C R Soc Biol (Paris)* 1938, **120**, 556

<sup>2</sup> Lipschütz A., and Iglesias R., *C R Soc Biol (Paris)* 1938, **120**, 519, *Iglesias R Public Med Exp (Chile)*, 1938

<sup>†</sup> Later we have found that fibroids can be induced by as little as 2% per injection or a total of only 80% of estradiol in the course of 3 months when the monocaprylic ester is used (Lipschütz Vargas, Baeza Rosales and Baeza Herrera, to be published soon)

<sup>3</sup> See Lipschütz, Vargas, Koref, Jedlicky, Bellolo, Murillo, Rodríguez, Chaume, Szabo Ruz, *C P Soc Biol (Paris)*, 1938-1939 **120**, 131, *The Lancet*, 1939-1940 *Public Med Exp (Chile)* 1939-1940, *Rev Chil Hist y Med Present* in press

<sup>4</sup> Lipschütz, A., and Vargas L., Jr, *The Lancet* 1939, **1**, 1313



## EXPLANATION OF FIGURES

In each figure explanation three data are given the first indicates the quantity per injection, the second the total amount and the third the duration of treatments

Fig 1 Estradiol monobenzoate 40 $\gamma$  (total 21 mg) 125 days Tumor on the

dorsal face of the uterus No sharp limit between tumor and myometrium  
Adenomatous condition of endometrium  $\times 5$

FIG 2 Estradiol monobenzoate  $20\gamma$  (total 5.8 mg) 183 days Three pedunculated tumors of uterus

FIG 3 Estradiol monobenzoate  $20\gamma$  (total 2.6 mg) 310 days Large subserous tumor with deformation of myometrium.  $\times 5$

FIG 4 Estradiol monobenzoate  $10\gamma$  (total 0.4 mg) 90 days Subserous uterine tumor Apical tumors on both horns the left one with ramifications covering left kidney and adhering to spleen, pancreas and abdominal wall

FIG 5 Estradiol monobenzoate  $10\gamma$  (total 0.4 mg) 108 days Chain of subserous tumors on the right horn, the chain corresponds to the muscular elevation present in the normal uterus Arrow points to small apical tumor

FIG 6 Estradiol monobenzoate  $10\gamma$  (total 0.4 mg) 91 days Mesometrial tumors

FIG 7 Estradiol monobenzoate  $80\gamma$  (total 2.4 mg) 85 days Apical tumors on both sides Partial necrosis of uterine horns (arrow)

FIG 8 Estradiol  $400\gamma$  (total 20.8 mg) 123 days No sharp limit between myometrium and mesometrial tumor Polyps in the uterine cavity  $\times 5$

FIG 9 Estradiol 17 benzoate 3 n butyrate  $10\gamma$  (total 0.4 mg) 91 days. A—"Fibroids" of the uterine submucosa Cystic glandular hyperplasia  $\times 5$   
B—Same preparation.  $\times 45$  No sharp limit between "tumor" and stroma of uterine mucosa

FIG 10 Estradiol monobenzoate  $20\gamma$  (total 2.6 mg) 310 days Uterine cervix surrounded by vagina Two vaginal fibroids, one pedunculated.  $\times 5$

FIG 11 Estradiol monobenzoate  $80\gamma$  (total 3.2 mg) 110 days Cystic dilatation of right tube with thick fibrous masses in the wall of the cyst Apical tumor on the left (upper half of left uterus cut away)

FIG 12 Estradiol monobenzoate  $40\gamma$  (total 2.1 mg) 124 days Tumor near cardia, and tumor of small curvature

FIG 13 Estradiol monobenzoate  $80\gamma$  (total 5.8 mg) 183 days Subserous tumors of the stomach

FIG 14 Estradiol monobenzoate  $80\gamma$  (3.0 mg) 88 days A—Tumor in the hilum of spleen (arrow) B—Mesenteric tumor (arrow 1) Fibrous strands in the mesentery (between arrows 2, 3 and 4 with small tumor of the intestine (arrow 3) C—Apical tumors, the right one enormous (arrow 2) Subserous uterine tumor (arrow 3) Tumor between diaphragm and abdominal wall (arrow 1)

FIG 15 Estradiol monobenzoate  $80\gamma$  (total 3.9 mg) 122 days Mesenteric tumor  $\times 5$

FIG 16 Estradiol monobenzoate  $10\gamma$  (total 0.4 mg) 90 days Tumors in the hilum of the spleen.

globe the upper third or more of the uterine horn (Fig 7) There is often no sharp limit between the myometrium and the tumor (Figs 1, 8) Sometimes the myometrium is deformed by the subserous tumor (Fig 3) The fibroids of the submucosa are still less delimited (Fig 9-B)

The subserous *extrauterine* tumors are to be found on most of the abdominal organs on the stomach (Figs 12, 13), spleen (Figs 14-A, 16), pancreas liver, kidney urinary bladder, in the epiploon, in the mesentery from the pylorus to the rectum (Figs 14-B, 15), on the abdominal wall (Figs 4, 13) and the diaphragm (Fig 14-C) Very frequently fibrous strands visible to the naked eye can be found on the abdominal wall, the mesentery and other parts of the abdominal cavity

*The uterine or extrauterine localizations have no connection with lymphatic glands*



There is no case with tumors in the thoracic cavity. Tumors present on the abdominal side of the diaphragm may infiltrate between muscle fibres, but the thoracic side of the diaphragm remains always smooth and free of fibrous nodules. No tumors are found at the site of injection and only very exceptionally at the site of subcutaneous implantation of a tablet of estradiol.

## 11838 P

## Studies on Fluorescence Associated with Proteins

WENDELL REEDER AND V. E. NELSON

*From the Laboratories of Physiological Chemistry, Iowa State College*

The relative fluorescence intensities of the proteins and their hydrolysates were determined by measuring the dilution required to reduce the fluorescence of a given amount of protein or protein hydrolysate to the same intensity as the fluorescence of a diluted standard solution of quinine bisulfate.

The proteins prepared and studied in this investigation were casein, wheat gluten, gliadin, glutenin, blood fibrin, gelatin, ovalbumin and zein. Hair and wool were also compared to the above proteins.

When examined in ultraviolet light of wavelengths 3100-4100 Å the proteins give a uniform bluish-white fluorescence in the solid state and a somewhat more green fluorescence in solutions. The fluorescence of these proteins is more green in basic solution than in acid, but the color change is not sharp.

Fluorescence of proteins is destroyed by oxidation with strong nitric acid or by ashing. The small amount of protein ash is not fluorescent in the solid state nor in acid, basic or neutral solution.

Organic solvents do not extract the fluorescent material from the solid protein nor from the protein hydrolysates in acid or basic solution. Likewise, dialysis experiments failed to remove the fluorescent material from protein solutions but after hydrolysis with strong acid the fluorescent material is readily removed from proteins by dialysis.

Hydrolysis of proteins by proteolytic enzymes or alkali produced only a slight increase in the amount of fluorescence. However, hydrolysis with hydrochloric acid, sulfuric acid, perchloric acid or phosphoric acid produced large increases in the fluorescence of those

proteins containing tryptophane and only slightly increased fluorescence in those proteins which are deficient in this amino acid. The color of the fluorescence produced during acid hydrolysis is blue-green.

Crude commercial proteins produce the same amount of fluorescence, during acid hydrolysis, as these same proteins prepared in a purified state. The presence or absence of air during acid hydrolysis does not affect the amount or color of fluorescence of the protein hydrolysates.

Nineteen amino acids were examined in ultraviolet light for fluorescence. These amino acids were not noticeably fluorescent in the solid state nor in acid, basic or neutral solution.

Amino acid additions to proteins during hydrolysis revealed that tryptophane was the only amino acid which increased the amount of fluorescence during acid hydrolysis. Tryptophane did not affect the amount of fluorescence produced during hydrolysis of proteins with alkali.

When proteins such as zein and gelatin, which are deficient in tryptophane, are hydrolyzed with acid in the presence of this amino acid the amount of fluorescence is greatly increased. However, when tryptophane is added to proteins containing this amino acid the fluorescence is only slightly increased during acid hydrolysis.

The addition of the vitamins which are capable of producing blue-fluorescent compounds did not affect the amount or color of fluorescence during acid hydrolysis.

Boiling acetic acid with proteins greatly increased the amount of fluorescence. However, the fluorescent color produced is much more blue than that of protein solutions or hydrolysates. Boiling acetic acid with several of the amino acids caused a blue fluorescence to appear in the solutions. Strong acids do not produce fluorescence of amino acids. This would indicate that the fluorescence increase with acetic acid and protein is due to a different substance than is produced during acid hydrolysis with strong acids.

Tyrosine and tryptophane were the only amino acids of all those studied which gave blue-green fluorescence with glucose when boiled with dilute HCl. The melanin produced by the action of the tyrosinase of potato juice on pure tyrosine solutions was not fluorescent.

Concentration of the fluorescent material was accomplished by adsorption of the material from acid solutions with English fuller's earth (Cenco fuller's earth was a poor adsorbent) and subsequent elution from this adsorbent with ethyl or methyl alcohol-ammonium hydroxide solutions. From its solubility properties the fluorescent

material was found to be different from the blue-fluorescent alkaloid harman produced by the mild oxidation of the acetaldehyde-tryptophane complex. Harman when injected intraperitoneally into rats caused paralysis of the hind legs of the animals and slowing of heart action whereas injection of neutralized protein hydrolysate or the adsorbed material from the hydrolysate had no effect.

Lactoflavin, thiochrome, quinine bisulfate and the fluorescent material from protein hydrolysates give broad bands of fluorescent light. The fluorescent spectrum of the material concentrated from casein hydrolysate is in the violet-green region of the spectrum and is very similar to the fluorescent spectrum of quinine bisulfate. The fluorescent spectra of lactoflavin and thiochrome are different from the fluorescent spectrum of protein hydrolysate. The fluorescent spectrum of the material concentrated from casein hydrolysate is in the violet-green region having wavelengths 4100-5300 Å. The fluorescence of protein hydrolysates is excited only by light of wavelengths 3400-3600 Å.

### 11839 P

#### Production of Pernicious Anemia-like Syndrome in Rats with Bile Acids

RAPHAEL ISAACS

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Because of the property of bile acids to produce hemolysis of red blood cells, cytolysis of leukocytes, destruction of nerve cells and myelin of nerve fibers, as well as to produce gastric lesions, a group of symptoms present in pernicious anemia, 12 rats were injected subcutaneously from one to 6 times a week with a suspension of glycocholic acid from ox bile. The effective daily dose after trials with weaker suspensions, was 10 mg. Of 12 rats, 3 developed a mild macrocytic, oval red blood cell anemia during the course of 2 months. During this time, one developed dragging of the hind legs, with a clumsy gait, and in 3 months, 2 showed skin ulcerations in regions which had not been used for injections. These symptoms did not appear in a control group, nor in a group injected with liver extract at the same time as the glycocholic acid.

Table I shows the nature of the blood changes.

The enlargement of the red blood cells appeared before any marked

TABLE I

Day	R.B.C millions per mm <sup>3</sup>	Mean corpuscular vol, $\mu^3$	R.B.C, size†
0	8.4	52.0	25 50 25
1*	8.5	52.0	
7	7.9	51.5	
14	8.8	53.0	
21	7.5	53.5	
28	7.8	55.2	11 34-55
35	7.0	58.8	
42	6.2	55.5	
49	4.5	61.0	
56	4.8	60.2	8 18 74
63‡	4.5	59.7	
70	6.5	60.5	

\*Suspension of 10 mg of glycocholic acid in water, 6 times a week

†Measurements less than 6.0 microns, 6.0 microns, larger than 6.0 microns

‡Liver extract injections begun

change in the red blood cell count was noted. An increase in the red blood cell count was noted 2 weeks after the subcutaneous injection of liver extract (7 units, once a week, for 2 weeks). The mean corpuscular volume was still elevated at the end of 2 weeks of therapy (reticulocyte increase).

The fact that but 3 of the 12 rats developed changes, suggests the continuation of the experiments with attention to variations in age of the animals, diet, size of dose, solubility of the preparation used, type of bile acid, frequency and amount of injections, state of the blood and bone marrow before injection, and histological condition of the stomach after the appearance of macrocytes (*cf* ulcer formation in guinea pigs).

The observations suggest the hypothesis that an increase or defect in the metabolism of bile acids in pernicious anemia causes a defect in the secretion of the "intrinsic factor", resulting in the development of a macrocytic anemia, and permitting an increase in hemolysis. The bile acids acting on the central nervous system, would cause the degenerative changes. Leukopenia would be another result.

From preliminary observations, it has been established that the bile acids are quite definitely increased in the blood in patients with pernicious anemia in relapse, confirming the report of Blankenhorn.<sup>1</sup>

*Summary.* A pernicious anemia-like syndrome has been produced in individual rats, characterized by oval macrocytosis and neurological changes following the subcutaneous injection of glycocholic acid.

<sup>1</sup> Blankenhorn, M. A., *Arch. Int. Med.*, 1917, 10, 344.

## 11840 P

## Differences Between High and Low Breast Tumor Strains of Mice When Ovariectomized at Birth \*

GEORGE WOOLLEY, ELIZABETH FEKETE AND C C LITTLE

*From the Roscoe B Jackson Memorial Laboratory, Bar Harbor, Maine*

The following differences were noted between 2 high and one low breast tumor strains of mice when ovariectomized at birth. The high breast tumor strains were the Jackson Laboratory dilute brown "JAX" (dba) and "JAX" C3H strains. These 2 strains have a high incidence of mammary tumors both as breeding and as virgin animals. The low tumor strain was the Jackson Laboratory "JAX" C57 black strain. The observations are based on a group of 125 JAX dba, 20 JAX C3H and 30 JAX C57 black mice. The results for each strain as reported in Table I have been uniform, and we believe deserve preliminary presentation, although not all of the above animals are dead up to the present time.

Easily noticed differences concerned the vagina. The vagina of the ovariectomized JAX dba and JAX C3H mice did not present the classic castrate picture but became much more stimulated than might have been expected. With both strains the vagina opened only a few days later than that of non-castrated littermate females. Progress to obvious mucification and to distinct cyclic cornification is much

TABLE I  
Findings for Animals Ovariectomized at Birth and Observed When Beyond One Year of Age

	High tumor		Low tumor
	JAX dba	JAX C3H	JAX C57
Vaginal orifice	Open	Open	Closed or small pore
Cyclic vaginal cornification	Yes	Yes	No
Uterine stimulation	"	"	"
Mammary gland	Extensive growth, marked development	Extensive growth, marked development	Variable growth, slight development
Mammary adenocarcinoma†	26	2	0
Nodular hyperplasia of adrenal cortex	Extensive	Extensive	Slight
Mating with male	No	No	No

†The dba and C57 Black strains have reached an older average age than the C3H strain at the present time and, therefore, no estimation of the final percentage of tumors can be attempted.

\* Supported in part by a grant from the Josiah Macy, Jr., Foundation

slower than with normal females. The vaginas of some of the JAX C57 black females failed entirely to open although observed from birth up to 20 months of age. Usually, however, a small pore appeared and remained more or less open.

The uterus of the JAX dba and JAX C3H mice after remaining small up to about 6 months of age began to enlarge and attained a size as large, and much larger in most cases, than those of virgin females of a comparable age. The uterine glands were enlarged and sometimes cystic. The JAX C57 black uterus was small and similar to that of a pre-puberty female throughout life.

The mammary glands of the high cancer strain females developed extensively and mammary adenocarcinomas appeared in ovariectomized females of both strains. With the low cancer strain some of the mammary glands remained rudimentary and comparable to those of normal male mice, while others, sometimes in the same mouse developed systems with long but slender ducts. No mammary adenocarcinomas were observed. Even so-called pre-cancerous changes were not present although frequent in the glands of the high tumor females.

The adrenal glands of the JAX dba and JAX C3H mice began to show grossly noticeable areas of nodular hyperplasia<sup>1</sup> in a few cells in the outer edge of the adrenal cortex around 3 months of age. A number of these areas eventually appeared and enlarged until the adrenal was uneven in contour and several times normal size. All animals had abnormal adrenals. In a few cases cortical carcinomas occurred and attained a size of one centimeter or more in diameter. In the JAX C57 black females very small areas of nodular hyperplasia also occurred in the adrenal cortex and in one case, at least, at around 3 months of age. Few of these areas appeared, however, and but little enlargement of them occurred in later life. The contour and size of the adrenal gland remained quite normal.

Males were experimentally placed with the females but so far no vaginal plugs have been found indicating that normal mating reactions were not attained.

Although the above differences were first noted with mice ovariectomized at birth, they have no necessary connection with early castration in itself. Marked similar strain differences appeared when the ovaries were removed at one month and at 6 months of age.

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<sup>1</sup> Woolley, G. W., Fekete, Elizabeth, and Little, C. C., *Proc. Nat. Acad. Sci.*, 1939, **25**, 277.

<sup>†</sup> Some of the early stages were found by William Daughaday, a holder of a Harvard University National Scholarship and a summer student at the Roscoe B. Jackson Memorial Laboratory.

*Conclusion* Marked differences appeared in strains of mice when they were ovariectomized at birth and then examined in later life. The high tumor JAX dba and JAX C3H strains showed stimulated uterus, vagina and mammary glands. The adrenal glands exhibited extensive nodular, hyperplastic areas. All of these organs of the low tumor JAX C57 black strain remained essentially unstimulated. To ascertain whether the above differences are characteristic of all high and low tumor strains, other strains are being studied.

11841

### Observations on Sensitization of Guinea Pigs with Alum-Precipitated Ragweed Extract

E J COULSON AND HENRY STEVENS (Introduced by A. K. Balls)  
*From the Bureau of Agricultural Chemistry and Engineering, U S Department of Agriculture*

Alum-precipitated ragweed pollen extract is a potent antigen for producing anaphylactic sensitization in guinea pigs,<sup>1-4</sup> whereas the watery extract is by comparison a poor one. Alum-precipitated extracts have been successfully applied in sensitizing guinea pigs to house dust<sup>5-8</sup> and cotton linters<sup>9, 10</sup> and in sensitizing dogs to horse serum.<sup>10</sup> The enhanced antigenic response of the alum-precipitated extract is generally attributed to two factors: first, retention of the antigenic substance by the precipitate so that it is only slowly liberated into the system, thus producing a continuous stimulation for the production of antibodies, and second, the local tissue response to the precipitate at the site of injection is said to delay absorption of the antigen. Thus diphtheria toxoid has been detected at the site of injection from a few days to several weeks after administration.

1 Harrison, W T, *U S Public Health Reports*, 1934, **49**, 462

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of alum-precipitated toxoid<sup>11 12</sup> The experiments recorded here were designed to determine whether antigenic constituents of ragweed pollen could be detected in the indurated nodule that forms at the site of injection of the alum precipitate in guinea pigs and persists for several months

*Experimental* An alum-precipitated ragweed pollen extract (1-20 dilution) was prepared in the manner previously described<sup>6</sup> For sensitizing, 1 ml of the whole alum-treated extract was employed for subcutaneous injection because it was found that the antigenic constituents were not completely precipitated by the alum Thus precipitates, separated by centrifugation from two 1 ml aliquots of the alum suspension, were suspended in 0.9% sodium chloride and injected subcutaneously into 2 normal guinea pigs The supernatant solutions, without further treatment, were likewise injected into 2 normal animals Thirty-four days later each animal received an intravenous injection of 1-100 ragweed extract in 0.9% sodium chloride The results are shown in Table I

Each animal sensitized with the alum precipitate was found to be fatally sensitized At autopsy indurated nodules were found at the site of the sensitizing injection The animals that had been sensitized with the supernatant solution from the alum-precipitated extract showed symptoms of anaphylaxis but were not fatally sensitized No nodule was present in either of these 2 animals at the site of the sensitizing injection

After an incubation period of 30 days guinea pigs sensitized with the whole alum-treated extract regularly respond with anaphylactic shock upon the intravenous administration of ragweed pollen extract Fourteen animals sensitized in this manner were injected intravenously with 0.1 ml of a 1-100 water extract of pollen Eight responded with immediate fatal anaphylaxis, 4 with severe anaphylaxis, and 2 with moderate anaphylaxis Of 29 animals sensitized in

TABLE I  
Sensitization with the Precipitate and Supernatant Solution from Alum Treated Ragweed Pollen Extract \*

Animal No	Sensitized with	Test injection,† ml	Symptoms
407	Precipitate	0.2	Fatal anaphylaxis, 3½ min.
409	"	0.2	" " " 4 "
406	Supernatant	0.2	Severe anaphylaxis
408	"	0.4	Slight

\*Period between sensitization and shock was 34 days

†Shock material was 1:100 aqueous pollen extract

<sup>11</sup> Glenny, A. T., Buttle, G. A. H., and Stevens, M. F., *J. Path. and Bact.*, 1931, **34**, 267

<sup>12</sup> Harrison, W. T., *Am. J. Pub. Health*, 1935, **35**, 298



## 800 SENSITIZATION WITH ALUM-TREATED RAGWEED EXTRACT

the same manner and injected with 0.05-0.1 ml of a 1-20 water extract of pollen 23 responded with fatal anaphylaxis and 6 with severe anaphylaxis.

To determine whether antigenic components of ragweed pollen remain unabsorbed in the indurated nodule that appears at the site of injection of the alum precipitate, the following experiments were performed.

Ten guinea pigs weighing between 261 and 347 g were sensitized with alum-precipitated ragweed extract. Thirty-three days later when 2 of the animals were tested for sensitivity by intravenous injections of 0.2 ml of 1-100 pollen extract both responded with fatal anaphylaxis in  $2\frac{1}{2}$  and 3 minutes, respectively. The remaining 8 animals were killed by a blow at the base of the skull and the nodules resulting from the sensitizing injection were dissected. Nodules from 2 or 3 sensitive animals were pooled, triturated in 0.9% sodium chloride and injected subcutaneously into 6 normal guinea pigs. In order to exclude the possibility of passive sensitization 2 of the animals were tested for sensitivity 4 days after injection with the nodules (Table II). Neither of these showed any symptoms of anaphylaxis. Both responded with fatal shock upon injection of pollen extract 28 days later. The 4 remaining animals at the end of the 32-day incubation period were all demonstrably sensitive to the ragweed extract. At autopsy nodules were noted in some of these animals but had disappeared in others.

*Discussion* The fact that it is possible to actively sensitize a second guinea pig by the material that is walled in by the cyst which forms at the site of injection of alum-precipitated ragweed extract indicates that the original antigenic substance must be present in practically an unchanged condition and that alum considerably delays the absorption. The continuous stimulus exerted by the slowly absorbed antigen therefore may account for the enhanced antigenic potency of the pollen extract.

TABLE II  
Sensitization with Indurated Nodules

Animal No	Sensitized with nodules from animals No	Incubation period, days	Test injection,* ml	Symptoms
866	760, 788, 789	4	0.2	None
		32	0.2	Fatal anaphylaxis, $3\frac{1}{2}$ min.
870	798, 802, 803	4	0.3	None
		32	0.2	Fatal " $3\frac{1}{2}$ min.
867	760, 788, 789	32	0.2	Slight " "
868	791, 792	32	0.2	Moderate " "
869	791, 792	32	0.2	Fatal " $3\frac{1}{2}$ min.
873	798, 802, 803	32	0.2	Fatal " 6 "
888	Control		0.5	None

\*Shock material was 1:20 aqueous pollen extract

## 11842 P

Variation in Immune Response to *Brucella abortus* Depending on Route of Administration

PAUL O HAGEMAN AND J A DOUBLY (Introduced by David P Barr)

*From the Department of Medicine, Washington University School of Medicine, St Louis*

A perplexing diagnostic problem is presented by the combination of vague complaints, low titer *Brucella* agglutination, and positive Brucellergen skin test. Whether such findings indicate past or present infection or whether they lack significance is a fundamental problem.

It was thought that the ingestion of milk containing *Brucella* organisms killed by pasteurization might be responsible for this confusion by stimulating the formation of agglutinins. This hypothesis seemed especially plausible, since heat-killed *B. typhosus* given orally and intravenously yields similar immunological responses, and particularly because of the similarity in the accepted pathogenicities of typhoid and undulant fevers. The investigation of the immune reactions to *Brucella abortus* following injections by these routes was undertaken.

*Technic* A stock culture of *Brucella abortus* (National Institute of Health strain No. 456) was grown in 1% dextrose beef infusion broth aerobically at 37°C for 48 hours. The flasks were then heated in a waterbath for 30 minutes at 60°C. Samples of the heated culture showed no growth after 4 days' incubation at 37°C.

Part A Three groups of 4 rabbits each were used.

Group I received the heat-killed organisms suspended in milk by stomach tube. After centrifuging the heated culture for 20 minutes at 3,000 r.p.m. discarding the supernatant fluid, the organisms were resuspended in the same volume of fresh sweet pasteurized whole milk.

Group II received the heated broth culture intravenously.

Group III served as controls, received no injections, were kept on a regular diet and bled at the same intervals as Groups I and II.

During the first week 0.5 cc of culture or its milk suspension equivalent was given for 3 successive days, in the second week 1 cc was given on 3 successive days, and so on, 1.5 cc in the third week, and 2 cc in the fourth week. Blood was drawn for agglutination tests with the homologous strain before the first dose of organisms was

TABLE I  
Agglutination Titers.

Rabbit Group		Days					
		1	Injections begun	7	14	21	31
Part A	I	0*	oral	0	0	0	0
	II	0	intravenous	1 1040†	1 5120	1 5120	1 4480
	III	0	—	0	0	0	0
Part B	I	0	oral‡	0§	0	0	0
	II	0	intravenous	1 640	1 1280	1 1280	1 1280

\*0 = No agglutination in dilution of 1 20

†Figures quoted are averages for the group

‡Oral dosage of organisms in Part B is 20 × that in Part A

§One rabbit in this group died of a milk pneumonia because the injection was given intrabronchially. Titer of 1 20 was present just before death.

given, and at weekly intervals thereafter, and again 10 days after the final dose had been given

**Part B** A second batch of heat-killed broth culture of *Brucella* was prepared as described above. Two groups of 4 rabbits each were used, one receiving by stomach tube the same volumes of a milk suspension of the heat-killed *Brucella* culture concentrated 20 times (i.e., 0.5 cc milk contained the organisms from 10 cc of the original culture), and the other receiving the heated culture intravenously in the same doses employed in Part A. Bleedings were made at the same intervals as in Part A.

**Results** See Table I

**Part A** Rabbits receiving heat-killed *Brucella* organisms by vein developed agglutinins for that organism, while the same number of organisms given orally failed to stimulate the formation of agglutinin response.

**Part B** Increasing by 20 times the number of organisms given orally failed to stimulate formation of agglutinins.

**Discussion** Data presented here clearly indicate that, under the conditions of this experiment, heat-killed *Brucella* organisms introduced into the intestinal tracts of rabbits do not lead to the formation of agglutinins. The antigen is either not absorbed or is altered sufficiently by digestive processes so that it loses its antigenicity. In view of these results, ingestion of heat-killed *Brucella* organisms would not seem important as a cause for low agglutination titers for this organism. Likewise attempts to immunize against this infection by oral administration of non-viable *Brucella* organisms would seem futile.

**Summary** Rabbits develop agglutinins for *Brucella abortus* following intravenous injections but fail to do so after sizeable doses are introduced into the stomach.

## Calcium in Acute Pancreatic Necrosis \*

HUGH A EDMONDSON AND IRVING A FIELDS (Introduced by  
E M Hall )

*From the Department of Pathology, University of Southern California School of  
Medicine, and the Laboratory of the Los Angeles County Hospital*

In acute pancreatic necrosis neutral fat is split into fatty acid and glycerol by the action of lipase. The glycerol is absorbed and the fatty acids combine with calcium to form soaps.<sup>1</sup>

Recently one of us (H A E) performed an autopsy on a patient who died with symptoms of shock and tetany. Extensive fat necrosis in and around the pancreas and fat embolism were observed. The carbon-dioxide combining power was 47 volumes %, so alkalosis could not have caused the tetany. Serum calcium was not determined antemortem. It occurred to us that possibly due to excessive formation of fatty acids and subsequently calcium soaps, enough calcium had been removed from the blood to cause tetany. On analysis a 2 g sample of pancreas and peripancreatic tissue contained 10.5 mg calcium or 525 mg per hundred g tissue. As there were at least 300 g of tissue so affected some 1500 mg of calcium may be assumed to have been present. This figure is slightly in excess of the total normal amount of calcium in the blood serum and the extracellular fluid combined. The total serum calcium may be estimated at 600 mg. There is about 3 times as much extracellular fluid as blood plasma.<sup>2</sup> Studies of transudates reveal an average calcium content of 7.5 mg<sup>3</sup> or an estimated total of 750 mg in the extracellular fluid.

No published accounts could be found of the total calcium in the lesions of acute pancreatic necrosis or of the serum calcium level in this disease.

We have since studied 2 additional cases which came to necropsy, in one (G C) the disease followed surgery and was of less than 24 hours' duration. In this case the entire pancreas and involved peripancreatic tissue was used for analysis. In the second (C L) all the tissue (2250 g) was ground 3 times and 3 separate samples were taken which contained almost identical amounts of calcium.

\* Aided by a gift from Ethel Mossman Jacobs

<sup>1</sup> Langerhans, R, *Firchows Arch f path Anat*, 1890, **122**, 252.

<sup>2</sup> Gamble, James L, *Bull Johns Hopkins Hosp*, 1937, **61**, 151.

<sup>3</sup> Green, C H., Bollman, J L., Keith, M M., and Wakefield, E G, *J Biol Chem*, 1931 **91**, 203.

TABLE I  
Calcium Content of Lesions in Acute Pancreatic Necrosis Compared with Normal Human Controls

	Pancreas Ca mg per 100 g wet tissue	Peripancreatic fatty tissue Ca mg per 100 g wet tissue	Total Ca mg in pancreas plus 2 kg of adjacent fatty tissue	
C.R. (control)	56	26	Estimated maximum	580
E.Q. "	53	23	" "	520
H.P. "	60	36	" "	780
F.H.	5250		" "	15000
G.C.	—	—		2000*
C.L.	1730	1520		17320

\*Disease of less than 24 hr duration

TABLE II  
Serum Calcium in Eleven Cases of Acute Pancreatic Necrosis (Clinical)

Name	Serum calcium per hundred cc of blood which represent time elapsed since onset of illness														Results tabulated on days	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14		
B.W.			7													
O.B.						8			94			94				
T.R.	96															
V.C.			83					97								
J.E.			87			84										
W.D.	103															
J.B.							79	77		74	76			134		
								77			81		101			
L.K.	95		91		94											
			85	85												
M.E.			97	97												
				94												
A.D.				97												
J.M.		9		89	88											

Three normal controls were also studied. The results are given in Table I. To give comparable figures for the controls an estimate was made of the amount of calcium in the pancreas (estimated weight 100 g) plus that in 2 kg of adjacent fatty tissue.

We have also studied the serum calcium (Table II) in 11 individuals who clinically were suffering from acute pancreatitis. In all of these the urinary diastase and/or blood amylase were greatly increased.

**Conclusions** As much as 1732 mg of calcium may be present in the lesions of acute pancreatic necrosis. A moderate fall in serum calcium levels in acute pancreatic necrosis may occur between the 3rd and 11th days of illness.

## Further Studies on Active Milk Influence in Breast Cancer Production in Mice \*

JOHN J BITTNER (Introduced by C C Little )

*From the Roscoe B Jackson Memorial Laboratory, Bar Harbor, Maine*

The purpose of this article is to summarize the published evidence and to present new material that the milk influence in inherited breast tumor production in mice is an actual and active influence

In this paper the term tumor or cancer signifies breast carcinoma and unless otherwise stated, all the females were used as breeders

Various experiments have been reported by the author<sup>1-16</sup> on the rôle played by the milk influence, genetic susceptibility and/or hormonal stimulation in the etiology of inherited breast cancer in mice Others have confirmed the work regarding the milk influence in different strains<sup>17-20</sup>

The analysis of the data also indicated that another type of breast carcinoma may develop in mice which apparently is not genetic since the progeny do not show tumors The milk influence is generally inactive in such animals and the other etiological factors are not clear No difference has been noted in the histological structure of these tumors

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\* Assisted by grants from the National Cancer Institute and the International Cancer Research Foundation

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TABLE I  
Calcium Content of Lesions in Acute Pancreatic Necrosis Compared with Normal Human Controls

	Pancreas Ca mg per 100 g wet tissue	Peripancreatic fatty tissue Ca mg per 100 g wet tissue	Total Ca mg in pancreas plus 2 kg of adjacent fatty tissue	
C.R. (control)	5.6	2.6	Estimated maximum	58.0
E.Q. "	5.3	2.3	" "	52.0
H.P. "	6.0	3.6	" "	78.0
F.H.	525.0		" "	1500.0
G.C.	—	—		200.0*
C.L.	178.0	152.0		1732.0

\*Disease of less than 24 hr duration.

TABLE II  
Serum Calcium in Eleven Cases of Acute Pancreatic Necrosis (Clinical)

Name	Serum calcium per hundred cc of blood which represent time elapsed since onset of illness														Results tabulated on days
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	
B.W.			7												
O.B.						8			9.4			9.4			
T.R.	9.6														
V.C.			8.3					9.7							
J.E.			8.7			8.4									
W.D.	10.3														
J.B.							7.9	7.7		7.4	7.6			13.4	
								7.7			8.1		10.1		
L.K.	9.5		9.1		9.4										
			8.5	8.5											
M.E.			9.7	9.7											
				9.4											
A.D.				9.7											
J.M.		9		8.9	8.8										

Three normal controls were also studied. The results are given in Table I. To give comparable figures for the controls an estimate was made of the amount of calcium in the pancreas (estimated weight 100 g) plus that in 2 kg of adjacent fatty tissue.

We have also studied the serum calcium (Table II) in 11 individuals who clinically were suffering from acute pancreatitis. In all of these the urinary diastase and/or blood amylase were greatly increased.

*Conclusions.* As much as 1732 mg of calcium may be present in the lesions of acute pancreatic necrosis. A moderate fall in serum calcium levels in acute pancreatic necrosis may occur between the 3rd and 11th days of illness.

# Further Studies on Active Milk Influence in Breast Cancer Production in Mice \*

JOHN J BITTNER (Introduced by C C Little)

*From the Roscoe B Jackson Memorial Laboratory, Bar Harbor, Maine*

The purpose of this article is to summarize the published evidence and to present new material that the milk influence in inherited breast tumor production in mice is an actual and active influence

In this paper the term tumor or cancer signifies breast carcinoma and unless otherwise stated, all the females were used as breeders

Various experiments have been reported by the author<sup>1-16</sup> on the rôle played by the milk influence, genetic susceptibility and/or hormonal stimulation in the etiology of inherited breast cancer in mice Others have confirmed the work regarding the milk influence in different strains<sup>17-20</sup>

The analysis of the data also indicated that another type of breast carcinoma may develop in mice which apparently is not genetic since the progeny do not show tumors The milk influence is generally inactive in such animals and the other etiological factors are not clear No difference has been noted in the histological structure of these tumors

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\* Assisted by grants from the National Cancer Institute and the International Cancer Research Foundation

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In the earlier papers it was stated that the incidence of breast tumor in mice ("A" stock) might be reduced from 90% (over 500 animals) to 8% (127 mice) by fostering the young of high breast tumor stock mothers to females of low breast tumor strains. The young were transferred before they were 24 hours old, and only one generation was fostered.

However, some of the young which were permitted to remain with their potentially tumorous mothers for less than 24 hours developed tumors as did some of their progeny. Other mice of the same fostered litter did not develop tumors and had descendants which likewise gave a low incidence of breast tumors, the incidence being similar to that observed in virgin females of the stock.

Fifteen young which were fostered not before from 1-5 days after birth gave a tumor incidence of 87%.

Twenty-seven young of fostered "non-breast cancerous" females were given to control (high cancer) "A" stock females the day of birth, tumors resulted in 89% of these mice. The fostered "non-breast cancerous" females nursed 29 of their progeny and no tumors were recorded.<sup>10</sup>

To determine if the milk influence was present only in the first milk secreted by a lactating female, some high tumor stock females were permitted to nurse their litters for 4 days before being used as foster mothers.<sup>6, 9</sup> The young fostered were BAF<sub>1</sub> hybrids (C<sub>57</sub> black or "B" ♀♀ × "A" ♂♂). When the high tumor stock females were used as foster mothers the same day their own litters were born, the fostered F<sub>1</sub> females had a tumor incidence of 87.3% (63 animals). Females used one day after their litters were born nursed 54 BAF<sub>1</sub> mice and these females showed a breast tumor incidence of 85.2%. Two mice in each group developed internal tumors and if they are not included the respective ratios were 90.2% and 88.5%. Twelve BAF<sub>1</sub> females were given to "A" stock females which had nursed for 4 days, all of the hybrids developed breast tumors.

Hybrid females (BAF<sub>1</sub>) which had maternal parents from a low breast cancer stock and paternal parents from a high breast cancer stock have a spontaneous breast tumor incidence of 2% if they are nursed by their maternal parents.<sup>13, 16</sup> The number of mice observed was 118. If inoculated with grafts of spleen or thymus from 4-week-old "A" stock donors, or lactating mammary tissue from older animals, the observed incidence was 21.7% for 46 mice. BAF<sub>2</sub> control breeding females (246 animals) had a tumor incidence of 0.6% and 11 inoculated mice of the same generation gave 18.2% cancer. Seventeen BAF<sub>2</sub> mice descended from inoculated BAF<sub>1</sub> females showed a mammary gland tumor incidence of

29.4%<sup>6 11 16</sup> The mice of the inoculated groups were older at the time of tumor development than the control animals

ABF<sub>1</sub> ("A" ♀♀ \ "B" ♀♀) females, totaling 122, nursed by "A" stock females showed an incidence of 94.3%, whereas hybrids which had fostered "A" stock (low tumor)<sup>†</sup> maternal parents gave 0.6% cancer. Another group of 37 ABF<sub>1</sub> females was taken from their high tumor stock mothers and given to inbred C<sub>57</sub>-black stock females with a resulting breast tumor incidence of 81% for 37 individuals. Similar mice nursed by "B" stock females which in turn had been suckled by low tumor BZF<sub>1</sub> by B♀ hybrids (2.6% cancer, 39 mice) gave 4.2% cancer for 48 mice. If "B" stock females which had been nursed by high tumor hybrids (BZF<sub>1</sub> by Z or C<sub>3</sub>H♀ — 79.1% cancer for 43 animals) were used as foster mothers for ABF<sub>1</sub> females, these mice developed tumors in 78.4% of the total or 51 animals.

C<sub>57</sub>-black stock mice when nursed by high tumor stock females ("A" or C<sub>3</sub>H) have a breast tumor incidence of approximately 10%<sup>15 17</sup>. The number tested was 104.

Ten mice of a sub-line of "A" stock fostered mice, having a tumor incidence of 4.5% for 245 breeding females, were fed an average of 5 cc of macerated liver per animal when they were 4 weeks of age. Tumors have not developed and the living animals are 16 months old. Ten additional females received by mouth an average of 1.7 cc of milk obtained from lactating females ("A" stock) 11 to 19 days after their litters were born. One animal died at 356 days of age, 8 have developed tumors, average age 276 days, and the remaining animal is living at 16 months of age. Ten other females, also 4 weeks of age, consumed an average of 0.9 cc of milk from the same source. Seven of the group are living (16 months) and 3 have developed tumors, average age 303 days. This work is being repeated and additional animals are under observation. The "A" stock donors (milk and liver) were from 92 to 155 days of age.

The injection of estrogenic hormones has failed to produce breast tumors in control C<sub>57</sub>-black stock mice<sup>21 22</sup>. Males of this strain nursed by females of the Paris R III high breast tumor stock will give rise to breast tumor following the implantation of crystals of estrone (Twombly<sup>22</sup>). Paris R III males nursed by C<sub>57</sub>-black stock

<sup>†</sup> Made by fostering "A" stock young to "B" stock females. The use of such animals eliminated the possibility of the hybrid young from obtaining milk containing the active influence.

<sup>21</sup> Sontzef, V., Burns, C. L., Moskop, M., and Loeb, L., *Am J Cancer*, 1936, 27, 229.

<sup>22</sup> Twombly, G. H., *Proc Soc Exp Biol and Med*, 1940, 44, 617.

females had a lower incidence of induced tumors than did the control animals

Pellets of theelin (average weight 0.2 mg) and estradiol benzoate (average weight 0.15 mg) were implanted subcutaneously into 6 males and 13 females of the "A" high tumor and 19 males and 24 females of the fostered "A" low tumor stock. Animals dying from too much hormone before the appearance of the earliest tumor, 147 days following injection, are not included. The results for the 2 hormones are combined as they were very similar. Six high tumor females and 3 high tumor males have developed tumors (47.4% of the total). The average tumor age for the females was 189 days and for the males 185 days following injection.

Eleven fostered "A" stock females and 12 males are still alive, all being over 330 days of age. The other females survived an average of 242 days and the deceased males 248 days. No tumors were observed in either group.

Three induced tumors have been noticed among 12 high tumor  $C_{3}H$  stock males and females whereas 18 fostered  $C_{3}H$  animals have been negative. Hybrids have also been injected with estrogenic hormones but many are still living. Tumors have appeared only in the animals which were nursed by high breast tumor stock females.

*Discussion* The foster-nursing experiments were started in an attempt to explain the maternal influence observed in breast tumor development in mice.<sup>23</sup>

Since 1936<sup>1</sup> it has been apparent that the incidence of breast tumors observed in high cancer strains of inbred mice might be reduced as the result of foster-nursing the young of such mothers by females of a low breast tumor strain. To account for these results it was assumed that a "breast cancer-producing influence" was transferred through the milk of breast cancer stock mothers to their progeny. Others stated however, that the absence of an active influence from the milk of cancerous strain females would produce similar results. The importance of the inherited breast cancer susceptibility factor and hormonal stimulation of the mammary tissue were emphasized in later work<sup>8, 9</sup> but need not be considered in detail here.

It was noted in the first experiments that if the fostered progeny of cancerous stock mothers were non-breast cancerous, few tumors were observed in their progeny and descendants. The progeny of "fostered non-cancerous" females nursed by cancerous stock females behaved as control or cancer stock individuals. If the fostered

<sup>23</sup> Staff, Jackson Memorial Laboratory, *Science* 1933, **78**, 465

females of a cancerous stock were themselves cancerous, breast tumors were common among their progeny

The importance of the time interval between birth and fostering was obvious when tumors were observed in the progeny of cancer stock mothers which were permitted to remain with their maternal parent longer than 24 hours. The incidence for young fostered within 24 hours after birth was low, the incidence for young fostered after 24 hours following birth approached the incidence recorded for the control animals. That the influence was active after the first milk had been secreted was manifested by the use of foster mothers of a cancer strain after they had nursed their own litters for 4 days.

Breast tumors have also resulted following the inoculation of lactating mammary gland tissue, spleen and thymus from donors of a high tumor stock and by feeding 4-week-old mice a small amount of milk obtained from lactating females 11 to 19 days after their litters were born. The incidence and average tumor age seemed to be determined by the amount of the active influence obtained by the animals. Macerated liver fed by mouth has failed to produce tumors although liver obtained from young donors has not been used.

Females of a low breast cancer stock when nursed by high tumor stock females ("A" or C H) have a breast tumor incidence of approximately 10%<sup>15, 17</sup>. The incidence is low, presumably, because they do not have the genetic breast cancer constitution. Fostered low breast tumor stock females do obtain the active milk influence when nursed by high tumor females and although they may not develop tumors, the influence may be transferred by nursing to other animals and tumors generally will develop in mice having the genetic breast cancer susceptibility.

The discussion of the relationship of the milk influence to induced estrogenic tumor induction must be delayed until further experimental data are available.

Thus all the evidence indicates an actual active influence in the milk of cancerous strain females. It is probably present during the entire lactation period as it is active in the first milk, in the milk obtained by nursing 4 days after the birth of a litter and in that secured 11 to 19 days after the beginning of lactation.

*Conclusions* The "breast cancer producing influence" is an actual active "influence" present in the milk of high cancer stock females. It is probably present and active during the entire lactation period.

The active influence may be transferred by the inoculation of spleen, thymus, and lactating mammary gland tissue from cancerous stock animals. The active influence probably is not present (or has been destroyed) in the liver of high tumor stock mice. The active influence may be given to 4-week-old females by feeding-by-mouth milk obtained from lactating females of a cancerous stock. Fostered females of low breast tumor strains need not develop breast tumors to transfer the active milk influence by nursing. An active milk influence may be necessary for the development of induced estrogenic breast tumors.

(A future publication will show that a low breast tumor strain of mice may "acquire" an active milk influence at any time, resulting in a high breast tumor strain.)

### 11845

#### Appearance of the Melanophore-Expanding Hormone of Pituitary Gland in Developing Chick Embryo \*

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The purpose of this investigation was to determine when the presence of the melanophore-dispersing hormone can first be demonstrated in the developing chick embryo with a view to correlating the appearance of the hormone with the structural development of the pituitary gland.

*Material and Methods.* Hybrid eggs (Red-Rock) were incubated for periods of from one to 13 days. In embryos of 5 days and over, the pituitary region was removed with the aid of a dissecting microscope. In the 2-, 3-, and 4-day embryos, the head of the embryo was utilized, and at the one-day stage the entire embryo was used. The material was ground up in a small volume of saline or 25% acetic acid together with a few drops of 2.5%  $\text{NaHCO}_3$  to bring the material to about pH 8. The resulting suspension was injected into the ventral sac of an hypophysectomized frog. The hypophyseal region of 6 to 20 chicks was used, depending on the age of the embryo.

Certain technical precautions were observed. Only frogs which had been hypophysectomized for at least 2 weeks were used, and the sensitivity of these was insured by testing them with minute doses.

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\* This investigation was supported by grant from the Committee on Research in Endocrinology, National Research Council.

of standard pituitary extract. Control injections of the acetic-bicarbonate mixture were made prior to injecting the suspension. Further control experiments were made with other tissues or regions of the embryo, and with extracts of acetone-desiccated powders, prepared from both the yolk and white of fertile and unfertile eggs. In no control case was a positive result obtained.

For histological study serial sections of the pituitary and surrounding structures of chick embryos ranging from 2 to 21 days' incubation were available.

*Observation.* Melanophore-dispersing activity could be elicited from the hypophyseal tissue of embryos of 5 days' incubation and in all subsequent stages. No attempt was made to estimate the exact amount present. However, it was found that in the 5-day-old embryos an extract prepared from 18 specimens gave a gross darkening of the frog which lasted for about 2 hours, while 9 and 6 specimens respectively gave a slight and a doubtful microscopic expansion of the melanophores.

Examination of serial sections of the chick embryo shows that the hypophysis undergoes considerable structural change between

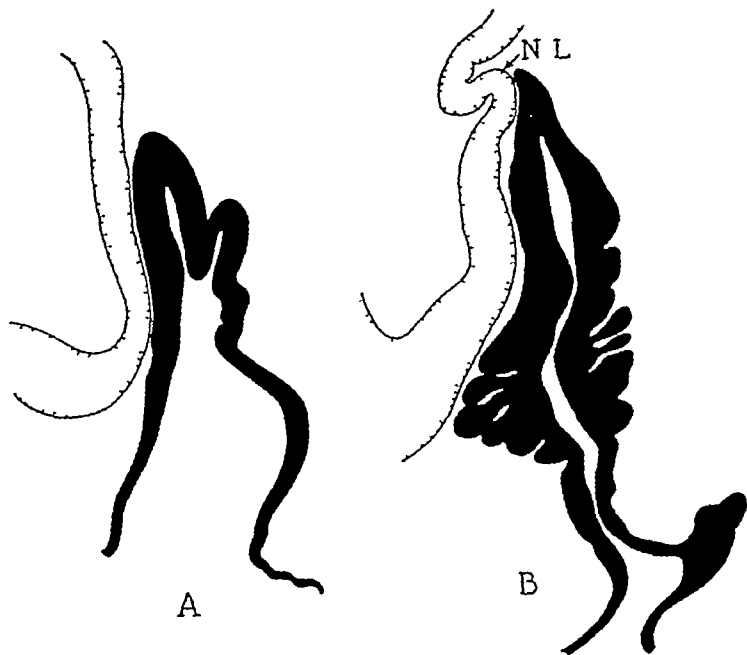


FIG. 1

A and B Pituitary gland in chick embryos of 4 and 5 days' incubation respectively. Solid black = anterior lobe. Stippled = brain and neural lobe (N.L.)

the fourth and fifth day of incubation (Fig 1) In the 4-day embryo the anterior lobe consists essentially of a wide evagination of the buccal epithelium, several ranks of nuclei in thickness There is no indication of a neural lobe In the 5-day embryo, the proximal end of the buccal evagination is constricted and attenuated, while the walls of the distal portion have become greatly thickened, with bud-like outgrowths extending into the surrounding mesenchyme The neural lobe appears for the first time as a small evagination of the infundibulum, which comes in close contact with the tip of the anterior lobe

*Discussion* The hypophyseal extracts prepared as described included varying amounts of neural lobe, brain, and mesenchyme in addition to anterior lobe tissue However, since in the adult chicken<sup>1</sup> the melanophore-expanding hormone is present in anterior but not in neural lobe extracts, it is assumed that in the chick the hormone arises in the anterior lobe

The melanophore-dispersing activity in the developing chick hypophysis precedes the appearance of granules in the cells of the anterior lobe From the above experiments the hormone appears first at the fifth day of incubation, while our own unpublished observations and those of Rahn<sup>2</sup> show that the cells first exhibit a general basophilic tendency at the eighth day and both basophils and acidophils appear at the tenth day This is in accord with similar observations on the pig embryo According to Snyder,<sup>3</sup> melanophore-dispersing activity is present in the 30 mm embryo, while Nelson<sup>4</sup> reports the appearance of basophils at 70-100 mm and of acidophils at 160-170 mm, coincidental with the appearance of growth and gonad stimulating activity respectively

Some investigators, notably Blount,<sup>5</sup> are of the opinion that in at least some species contact with neural tissue is necessary for differentiation and functioning of the intermediate lobe, which elaborates the melanophore-dispersing hormone in those species which possess an intermediate lobe It may therefore be of significance that melanophore-dispersing activity is not demonstrable in the chick hypophysis until the neural lobe has appeared Against this, however, is the fact that in the adult chicken the concentration of the melanophore-dispersing hormone is lowest in the juxta-neural

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<sup>1</sup> DeLawder, A. M., Tarr, L., and Geiling, E. M. K., *J Pharm and Exp Therap*, 1934, **51**, 142

<sup>2</sup> Rahn, H., *J Morph*, 1939, **64**, 483

<sup>3</sup> Snyder, F. F., *Am J Anat*, 1928, **41**, 399

<sup>4</sup> Nelson, W. O., *Am J Anat* 1933, **52**, 307

<sup>5</sup> Blount, R. F., *J Exp Zool*, 1932, **63**, 113, *Anat Rec*, 1939, **73**, Sup I, 7

region of the anterior lobe,<sup>1</sup> and, furthermore, the changes in the anterior lobe itself are sufficiently marked to account for the appearance of the hormone in the 5-day but not in the 4-day chick embryo

It is proposed to extend this work to include a study of the appearance of the pressor and oxytocic hormones in the pituitary of the developing chick embryo

*Conclusion* The melanophore-dispersing hormone is first detectable in the developing chick embryo of 5 days' incubation. At this stage, the anterior lobe no longer appears as a simple evagination of the buccal epithelium. The fact that the neural lobe first appears at this stage may or may not be of significance.

## 11846

### A Method of Preparing Isolated Intestinal Loops in the Dog \*

MILDRED E. DOSTER-VIRTUE AND ROBERT W. VIRTUE

*From the Department of Chemistry, University of Denver, Denver, Colorado*

Intestinal absorption experiments were carried on for 2 years with open fistulous loops of the Thiry type as modified by Johnston.<sup>1</sup> The double balloon method as adopted by Riegel<sup>2</sup> was also used for some months. Our results were not satisfactory as leakage often occurred and the animals required too much attention during experimental periods.

Therefore, a new operative method of closing intestinal fistulae has been developed and used during the past 2 years.<sup>†</sup> Present results indicate it to be successful physiologically, and superior experimentally to former procedures for constructing isolated intestinal loops for absorption studies.

A cylinder of especially prepared bone is implanted at the proximal end of the loop so that granulation tissue invades the interstices of the cancellous bone and forms a sealed closure for the loop.

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<sup>1</sup> Kleinholz, L. H., and Rahn, H., *Anat. Rec.*, 1940, **76**, 151.

\* A portion of this work has been done at the Medical School of the University of Colorado by the courtesy of Dr. R. W. Whitehead.

<sup>1</sup> Johnston, Chas. G., *Proc. Soc. Exp. Biol. and Med.*, 1932, **30**, 193.

<sup>2</sup> Riegel, Cecelia, Elsom, K. O., and Ravdin, I. S., *Am. J. Physiol.*, 1935, **112**, 669.

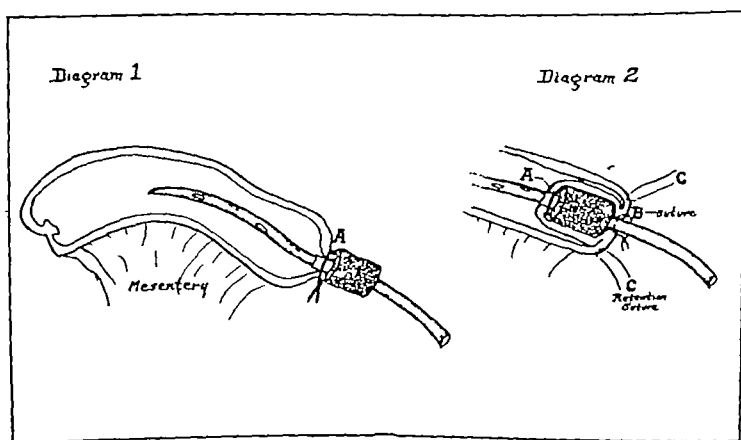
<sup>†</sup> This work was aided by a grant from the New Orleans Academy of Sciences.



Previously reported work with bone transplants has been done by Orell,<sup>3, 4</sup> Bisgard,<sup>5, 6</sup> Albee,<sup>7</sup> Wurm,<sup>8</sup> Girliani,<sup>9</sup> and Pokotilo<sup>10</sup>

Female dogs varying from 10 to 25 kg in weight have been used for the following surgical procedure. Six to 9 inches of small intestine are isolated for the loop, and the distal end is closed. The intestinal tract is anastomosed by a side-to-side apposition in the smaller animals and an end-to-end in the larger ones, by usual surgical methods. The open end of the loop is then inverted about the cylinder of bone which has been prepared and sterilized with a size 14 catheter through it. The accompanying diagrams show this procedure. Sutures hold the bone cylinder invaginated into the end of the loop so the serosal layer is held snugly to the surface of the bone. The catheter is brought out through the right rectus muscle, and the loop is anchored by retention stitches.

The work of Orell,<sup>11, 12</sup> Ghormley,<sup>13</sup> and Tavernier<sup>14</sup> on heterogeneous bone transplants and implants suggested some methods of



- 3 Orell, S, *Acta Chir Scandinav*, 1934, 74, 1
- 4 Orell, S, *Surg Gynecol and Obstetrics*, 1934, 59, 638
- 5 Bisgard, J D, *Arch Surg*, 1935, 30, 748
- 6 Bisgard, J D, and Farris, J M, *Surg, Gynecol and Obstetrics*, 1938, 66, 173
- 7 Albee, T H, *Proc Royal Soc Med*, 1930, 23, 31
- 8 Wurm, H., *Verhandl d deutsch path Gesellsch*, 1930, 25, 191
- 9 Girliani, G, *Arch Ital de Chir*, 1932, 31, 268
- 10 Pokotilo, K. L., and Kosdoba, A S, *Mitt a d Grenzgeb d Med u Chir*, 1936, 44, 390
- 11 Orell, S., *J Bone and Joint Surg*, 1937, 19, 873
- 12 Orell, S., *Surg, Gynecol and Obstetrics*, 1938, 66, 23
- 13 Ghormley, R H., and Stuck, W G, *Arch Surg*, 1934, 28, 742
- 14 Tavernier, *Lyon Chir*, 1930, 27, 233

treating osseous tissue for surgical uses. Our modified procedure is to use the solid areas of femora or humeri of cattle and cut them into small cylinders about one-half inch long. These pieces of raw bone are placed in 5% alkali overnight, washed with water for one hour, placed in acetone for one hour, and dried thoroughly. Size 14 catheters are cemented into the cylinders by a special rubber cement ‡. The lower end of the bone is also coated with it to seal the porous opening and stop any possible seepage through the cylinder. Ordinary rubber cement and several common plastics disintegrated in from one to 8 weeks in the animal body.

In 10 dogs autopsied or reoperated for removal of the loops, the specimens were studied microscopically. A sealed union of tissue into bone was always present macroscopically. Two microphotographs are presented that demonstrate this invasion of granulation tissue into the scaffolding offered by the bone matrix, and later a definite epithelialization from the serosa into the minute channels of the osseous cylinder. Thus, a combined organic and mechanical

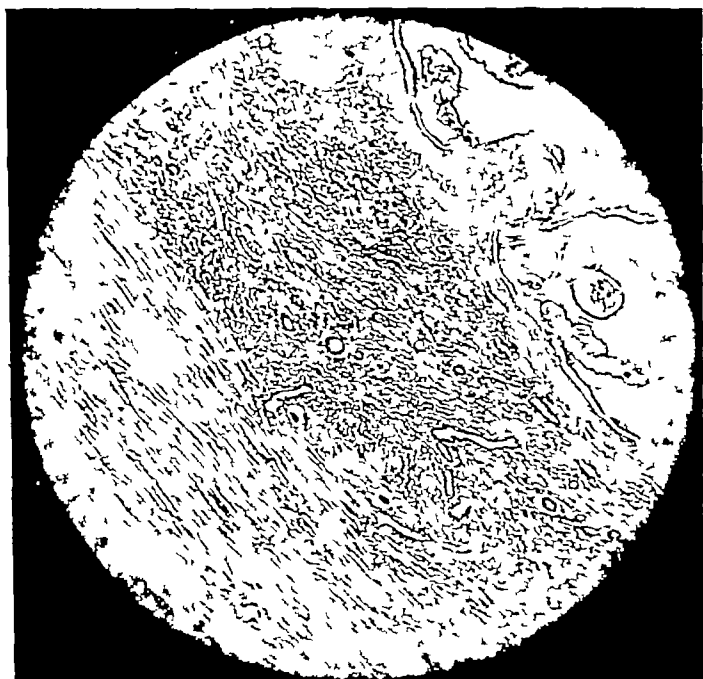


FIG 1

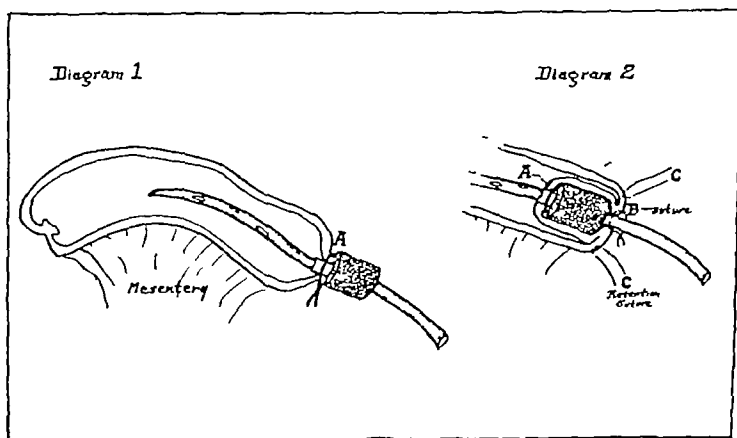
Cross section magnification ( $\times 40$ ) of junction of bone and intestinal serosa, 10 days after operation, showing vascular granulation tissue

‡ Gates Rubber Company, No 8675

Previously reported work with bone transplants has been done by Orell,<sup>3</sup> Bisgard,<sup>5</sup> Albee,<sup>7</sup> Wurm,<sup>8</sup> Girhani,<sup>9</sup> and Pokotilo<sup>10</sup>

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<sup>3</sup> Orell, S, *Acta Chir Scandinav*, 1934, 74, 1

<sup>4</sup> Orell, S, *Surg Gynecol and Obstetrics*, 1934, 50, 638

<sup>5</sup> Bisgard, J D, *Arch Surg*, 1935, 30, 748

<sup>6</sup> Bisgard, J D., and Farris, J M, *Surg, Gynecol and Obstetrics*, 1938, 60, 173

<sup>7</sup> Albee, T H, *Proc Royal Soc Med*, 1930, 23, 31

<sup>8</sup> Wurm, H, *Verhandl d deutsch path Gesellsch*, 1930, 25, 191

<sup>9</sup> Girhani, G, *Arch Ital de Chir*, 1932, 31, 268

<sup>10</sup> Pokotilo, K. L., and Kosdoba, A S, *Mitt a d Grenzgeb d Med u Chir*, 1936, 44, 390

<sup>11</sup> Orell, S, *J Bone and Joint Surg*, 1937, 19, 873

<sup>12</sup> Orell, S, *Surg, Gynecol and Obstetrics*, 1938, 60, 23

<sup>13</sup> Ghormley, R H., and Stuck, W G, *Arch Surg*, 1934, 28, 742

<sup>14</sup> Tavernier, *Lyon Chir*, 1930, 27, 233

treating osseous tissue for surgical uses. Our modified procedure is to use the solid areas of femur or humerus of cattle and cut them into small cylinders about one-half inch long. These pieces of raw bone are placed in 5% alkali overnight, washed with water for one hour, placed in acetone for one hour, and dried thoroughly. Size 14 catheters are cemented into the cylinders by a special rubber cement <sup>†</sup>. The lower end of the bone is also coated with it to seal the porous opening and stop any possible seepage through the cylinder. Ordinary rubber cement and several common plastics disintegrated in from one to 8 weeks in the animal body.

In 10 dogs autopsied or reoperated for removal of the loops, the specimens were studied microscopically. A sealed union of tissue into bone was always present macroscopically. Two microphotographs are presented that demonstrate this invasion of granulation tissue into the scaffolding offered by the bone matrix, and later a definite epithelialization from the serosa into the minute channels of the osseous cylinder. Thus, a combined organic and mechanical

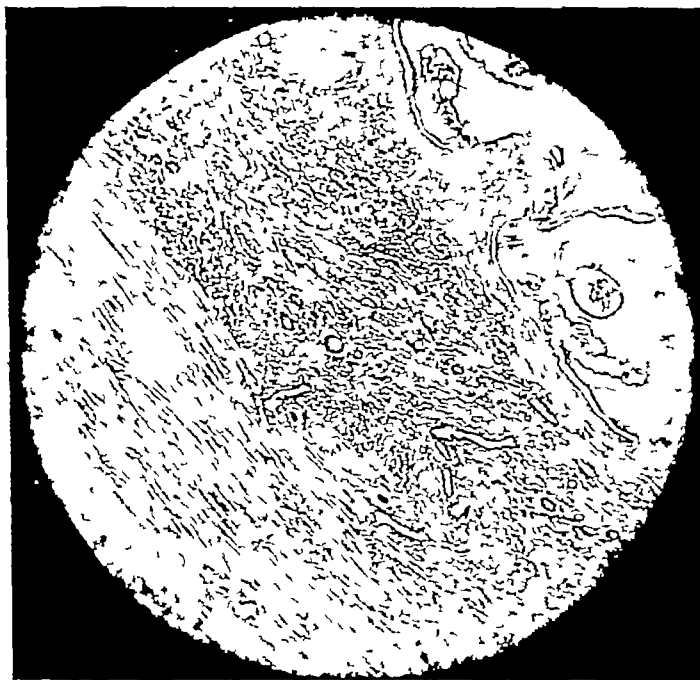


FIG. 1.

Cross section magnification ( $\times 40$ ) of junction of bone and intestinal serosa, 10 days after operation, showing vascular granulation tissue.

<sup>†</sup> Gates Rubber Company, No. 8675



FIG 2

Cross section magnification ( $\times 25$ ) of junction of bone and intestinal serosa, one month after operation, with granulation tissue becoming dense and fibrous

stopper is made at the fistulous openings of these intestinal loops

About 10 days after operation, experimental absorption or excretion periods are begun. The loop is irrigated daily with tap water, and all materials to be studied are easily introduced by syringes. The ease of working on this type of loop is readily appreciated after one has used the former types of fistulae. As needed, the catheter is closed by a Hoffman clamp, but usually it is left connected to a rubber football bladder that is held beneath the belly by a special metal pan that is strapped to the dog. This closed drainage is a great aid in cleanliness of the animals, and the metal band keeps the dogs from chewing the catheters.

A rubber disc is cemented about the catheter at the skin surface to immobilize it so that over a period of months very little tissue irritation occurs. Experimental absorption studies on these loops will be reported elsewhere.

Thus we present a new operative method of constructing an isolated intestinal loop that allows a convenient, clean, and leak-proof closure of the fistula.

## Anaerobic Uptake of Potassium by Frog Muscle

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Steinbach<sup>1</sup> has shown that frog muscle loses potassium in exchange for sodium when the muscle is soaked in potassium-free Ringer's solution. He also showed that when potassium depleted muscles are placed in Ringer's solution containing potassium they regain potassium at the expense of sodium. This uptake of potassium in exchange for sodium requires work which must be supplied by the muscle. It has previously been shown that muscle retains its potassium in Ringer's solution in the absence of oxygen.<sup>2</sup> The present investigation was undertaken to determine the influence of aerobic respiration on the uptake of potassium under the conditions of Steinbach's experiments.

The sartorius, semitendinosus and tibialis anticus longus muscles from recently caught frogs were dissected and placed in potassium-free Ringer's solution containing M/3000 phosphate buffer pH 7.4. After one hour the muscles from one frog were transferred to 250 ml of K-free Ringer's solution which was stirred by a current of air and kept in a cold room at 4°C. After about 17 hours the muscles were divided into sets for further treatment. This treatment was of 3 sorts, (a) immediate analysis (b) analysis after immersion for 4 or 7½ hours in 25 ml of Ringer's solution containing 10 mM K and M/150 phosphate buffer and continuously equilibrated with air (c) the same as (b) but continuously equilibrated with pure nitrogen starting ½ hour before the muscles were introduced. Paired sets of muscles were used to compare treatments (a) and (b) (b) and (c) and (a) and (c) as shown in Table I. Fenn and Cobb<sup>3</sup> state that muscles lose more potassium in Ringer's solution containing M/3000 phosphate than in M/150 phosphate. One incomplete experiment showed no difference in the uptake of potassium from solutions containing M/3000 and M/150 phosphate buffer.

On removal from the solution the muscles were individually blotted weighed on a torsion balance and placed in small weighing bottles. The bottles were dried over night at 100°C and the loss in

<sup>1</sup> Steinbach, H. B., *J Biol Chem.*, 1940, **133**, 695

<sup>2</sup> Dean, R. B., *J Cell and Comp Physiol.*, 1940, **15**, 189

<sup>3</sup> Fenn, W. O., and Cobb, D. M. *J Gen Physiol* 1934, **17**, 629

weight was taken as the water content. On small muscles weighing less than 150 mg this procedure is accurate to within 1%. Chloride was determined by a new micro-diffusion method in which HCl gas is liberated from the dry tissue by concentrated  $H_2SO_4$  and absorbed by NaOH on a piece of filter paper fastened to the lid of the weighing bottle. Chloride was determined on the paper by a modification of the method of Van Slyke.<sup>4</sup> The method and its errors will be described elsewhere. Potassium was determined on the sulphuric acid digest by the method of Wilde.<sup>5</sup> In this way determinations of water, chloride, and potassium could be made on the same tissue. The significance of the results was tested by Students' test, taking a probability that the sets are equal of less than 0.05 as significant.

Table I contains values averaged by sets for the results obtained on 10 frogs during the month of August.  $K_m$  and  $Cl_m$  are reported in micromols per gram of whole muscle. The fiber water was calculated on the assumption that all the chloride is extracellular and at a concentration of 114 millimolar.  $K_f$  represents the concentration of potassium in the fiber water. It is calculated from  $K_m$  by deducting the potassium in the extracellular phase which is assumed to have a concentration equal to that of the potassium in the Ringer's solution. A fiber potassium concentration,  $K_e$ , has also

TABLE I  
Effect of Oxygen and Nitrogen on Intake of Potassium from a High Potassium Solution

Set	Gas	Hr in 10 mM K	No of muscles	Dry wt, $H_2O$ mg %		$K_m$	$Cl_m$	Fiber $H_2O$ %	$K_f$	$K_e$	$K_d$
a	control	0	5	39.6	81	59	26	58	105	105	302
b	$O_2$	7½	5	39.2	81	69	36	49	137	125	355
a	control	0	8	27.8	81	62	33	52	120	120	326
c	$N_2$	7½	8	26.5	83	66	38	49	128	119	354
b	$O_2$	7½	9	20.3	82	63	50	39	152	141	329
c	$N_2$	7½	9	21.6	83	62	46	43	135	126	346
b	$O_2$	4	6	25	81	76	33	52	139	128	383
c	$N_2$	4	6	25	82	70	35	51	128	118	377

Average values for small isolated frog muscles. All concentrations are in micromols per gram.  $K_m$  and  $Cl_m$  are calculated per gram net weight.  $K_f$  is concentration in the water of a chloride free fiber.  $K_e$  is concentration in the water of chloride permeable fiber.  $K_d$  is amount per gram dry weight. Pairs of values in bold face type are significantly different.

<sup>4</sup> Van Slyke, D. D., *J. Biol. Chem.*, 1923, **58**, 523

<sup>5</sup> Wilde, W. S., *J. Biol. Chem.*, 1939, **128**, 309

been calculated on the assumption that chloride penetrates the fiber and has a concentration in the fiber water equal to the potassium concentration in the Ringer's solution. This assumption follows from the theoretical treatment of Conway and Boyle<sup>6</sup> (see Dean<sup>7</sup>) for fibers that are permeable to chloride as is probably the case when the external potassium is as concentrated as 10 millimolar. A potassium content per gram of dry weight has also been calculated,  $K_d$ , allowing for potassium in the extracellular spaces. This was calculated for a chloride-free fiber, values of  $K_d$ , calculated on the assumption that the fiber is chloride permeable, are slightly greater in set c. Since the dry weights show no consistent variation between paired sets, they probably are a good indication of the initial weights which were unfortunately not recorded.

The values of  $K_d$ ,  $K_e$ , etc., were calculated individually for each muscle and the results averaged. If the values of these concentrations are calculated from the average values of  $K_m$ ,  $Cl_m$ , and  $H_2O$  somewhat different results will be obtained because of the variability of the original data.

On the basis of potassium content  $K_m$ , there is no significant difference between the sets. However, the concentration of potassium inside the fibers  $K_i$  or  $K_e$ , is significantly greater in set b in air than in the initial set a, or the anaerobic set c. There is no significant gain in the concentration of fiber potassium in nitrogen. However, when we consider the amount of potassium in the fiber,  $K_d$ , it is apparent that after  $7\frac{1}{2}$  hours that the fibers in nitrogen have gained more potassium than either of the other two sets, and after 4 hours the gain is only 2% less in nitrogen than in oxygen. This paradox is explained by the fact that the muscles in nitrogen swell considerably and an increase in fiber size requires an uptake of potassium to keep the concentration constant.

This work shows that muscle fibers can take up potassium against a concentration gradient anaerobically as well as aerobically, and supports the previous work,<sup>2</sup> where it was shown that muscle fibers are permeable to potassium yet maintain their normal potassium concentration in nitrogen just as in oxygen. The fact that the fibers in nitrogen failed to increase their potassium concentration, might be explained by supposing that the fibers were already doing all the work that was possible just to maintain their potassium concentration as the fiber increased in volume.

<sup>6</sup> Conway, E. J., and Boyle, P. J., *Nature*, 1939, 144, 709

<sup>7</sup> Dean, R. B., *Biol. Symposia*, 1940, in press



## Failure of Thyrotropic Pituitary Hormone to Prevent Spontaneous Mammary Cancer in Mice

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Since Lacassagne proved that mammary carcinoma can be induced in mice by means of estrogens, workers in several laboratories have sought to discover what role, if any, pituitary hormones play in the development of this disease. The close relationship of ovary and pituitary gland in other biological processes makes this kind of research reasonable.

Cramer and Horning, in the laboratory of the Imperial Cancer Research Fund in London, gave a series of female mice of the RIII strain thyrotropic pituitary hormone subcutaneously, and reported<sup>1</sup> that they were able to prevent the development of mammary carcinoma in this strain by this means. They used a watery solution of pituitary hormone, sold under the trade name of *Ambimon*\*. It contained, according to biological assay, 150 guinea pig units of thyrotropic hormone and 50 units of gonadotropic hormone per cubic centimeter. This preparation was injected twice weekly in doses of 0.1 cc into the subcutaneous tissues of the mice. Only 19 mice were used. Their treatment was begun when they were 2 months of age. It was still being continued when Cramer and Horning wrote their report about a year later. At this time none of the mice had developed mammary carcinoma, and the authors felt justified in concluding that the development of the disease had been prevented by the hormone. Although these female mice had been kept with males, none of them had littered during the period of treatment. In a control series of 22 untreated female mice, which although kept with males had borne no litters. Cramer and Horning observed 11, or 50%, develop mammary carcinoma.

We have been able to repeat this experiment on a somewhat larger scale, using the same strain of mice and the same preparation of pituitary hormone. The RIII strain of mice has been bred by brother and sister matings in our laboratory since 1935, when we obtained 6 members of the strain directly from Lacassagne in Paris.

<sup>1</sup> Cramer, W, and Horning, E S, *Lancet*, 1938, 1, 72

\* Purchased from the N V Organon Co, Oss, Holland

Our control material now includes some hundreds of animals. The pituitary hormone was purchased from the same firm that supplied the London authors. We realized that the preparation was not a very pure one containing as it did 50 units of gonadotropic hormone in addition to 150 units of thyrotropic hormone per cubic centimeter. Since we were trying to repeat the London authors' experiment we had no choice except to employ it.

We treated a total of 33 mice. Of these 29 survived for at least 6 months, the lower age limit for the development of spontaneous mammary carcinoma in our control series. The treatment was begun when the mice were just under 2 months of age, 0.1 cc of the hormone being given twice weekly. It was continued for fifteen months. At the end of this time 13 mice remained alive. Our supply of hormone being exhausted, and its failure to prevent mammary carcinoma being apparent, the treatment was discontinued. All of the mice in the series are now dead and we are able to present the complete data. The fate of the 29 mice is shown in Table I.

Among these 29 treated mice that survived 6 months 16 therefore

TABLE I.

Mouse No	Age (months) treatment began	No Litters	Age (months) treatment ended	Age (months) death	Mammary carcinoma
801	1.6	0	9.7	9.7	0
803	1.7	2	14.5	14.5	+
805	1.6	0	16.3	27.0	0
807	1.5	3	11.3	11.3	+
809	1.6	0	14.7	18.0	0
815	2.1	1	15.7	16.4	+
817	1.9	0	15.5	25.4	0
819	1.8	0	8.9	8.9	0
821	1.9	1	15.1	15.1	+
823	1.9	0	16.7	31.3	0
825	1.8	0	14.8	19.3	0
827	1.7	0	15.8	15.8	+
829	1.6	0	14.6	16.9	+
831	1.7	0	7.2	7.2	0
833	1.2	0	10.8	10.8	+
835	1.4	2	14.6	29.2	0
837	1.3	2	15.5	15.5	+
841	1.5	0	14.5	17.0	0
843	1.4	1	14.3	19.8	+
847	2.0	1	16.1	26.0	0
849	1.5	0	16.1	20.8	0
851	2.1	3	10.5	10.5	+
853	1.4	3	10.5	10.5	+
855	1.7	2	9.4	9.4	+
857	1.6	0	13.9	24.0	0
859	1.6	2	8.5	8.5	+
861	1.7	2	10.3	10.3	+
863	2.0	1	12.9	12.9	+
865	1.8	0	9.8	9.8	+

developed mammary carcinoma. This tumor incidence is not significantly different from that for our untreated, unbred controls, and slightly lower than that for our untreated bred controls. In the light of our knowledge that breeding increases the incidence of spontaneous mammary carcinoma in this strain of mice this result might be expected, for the breeding record of this group of *Ambion*-treated mice was intermediate. Only 14 of the 29 bore litters, averaging 1.8 litters each. In our untreated control series of bred females 128 of the 140 that survived 6 months bore litters, averaging 5.5 litters each. Our conclusion must be that the treatment with *Ambion* prevented, to some extent, pregnancy in our mice, and therefore lowered slightly the incidence of mammary carcinoma. It certainly did not abolish the disease as Cramer and Horning claimed.

Histological study of the adrenals, ovaries, uterus, mammary glands, and mammary carcinomas when they developed, in these treated mice showed no abnormality which we have not found in the tissues of our control animals. In this strain of mice certain changes in the endocrine glands, which might be called degenerative, occur spontaneously. The thyroids were studied with special care, but the only difference between those of the treated animals and the controls that could be made out was a slightly greater cellularity of the glands in some of the former. The pituitary showed no gross changes in the treated mice. We were not able to carry out adequate histological study of this organ.

The statistical data for the treated animals and our controls is given in Table II.

Since 1938, when Cramer and Horning published their report of the carcinoma *inhibiting* effect of anterior pituitary, and we under-

TABLE II.

	Mice treated with thyrotropic hormone	Unbred control mice	Bred control mice
Total number surviving 6 mo	29	94	140
Developed mammary carcinoma	16 or 55.2% ± 9.23%	49 or 52.2% ± 5.15%	103 or 73.6% ± 3.72%
Mean age at death of those with carcinoma	13 mo ± 7.9	16.44 mo ± 7.3	12.15 mo ± 3.6
Median age at death of those with carcinoma	12.1 " ± 9.8	16.5 " ± 9.1	11.3 " ± 4.5
Standard deviation of age at death of group with carcinoma	3.15 " ± 5.6	5.12 " ± 5.2	3.67 " ± 2.5

Note. All corrections are standard deviations

took to verify their findings, Loeb and his associates<sup>2</sup> have reported that the same organ, when transplanted subcutaneously, causes the carcinoma rate to be *increased*. We have not attempted any transplantation experiments, but our data concerning the results of subcutaneous injection of the hormone may apply to some extent to Loeb's new interpretation of anterior pituitary function. Cramer<sup>3</sup> has presented an elaborate theory of the genesis of mammary carcinoma in which the pituitary takes an important rôle as an antagonist of the disease. His theory would appear to be premature.

*Summary* Long continued subcutaneous injection of an anterior pituitary preparation which contained thyrotropic hormone and some gonadotropic hormone did not definitely affect the incidence of mammary carcinoma in female mice of the RIII strain.

# 11849 P

## Typhus Rickettsia Isolated from Mice and Mouse-Fleas During an Epidemic in Peiping \*

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It is now generally recognized that "endemic" or murine typhus has its reservoir in rats and is transmitted to man by rat-fleas, particularly *Xenopsylla cheopis*<sup>1 2</sup>. However, in South Queensland Australia, circumstantial evidence strongly incriminates mice, rather than rats, as the reservoir, although actual proof of infection among these rodents or their ectoparasites is as yet lacking<sup>3 4</sup>. Lépine and Lorando,<sup>5 6</sup> in Athens, examining rats as well as mice trapped in the premises where cases of typhus had occurred, found that whereas rats were frequently infected with typhus Rickettsia, mice were

<sup>2</sup> Loeb, L., and Kirtz, M. M., *Am J Cancer*, 1939, **36**, 56

<sup>3</sup> Cramer, W., *Am J Cancer*, 1940, **38**, 463

\* We are indebted to Dr. A. B. D. Fortuyn for identification of the species of mice and to Dr. H. F. Hsü for that of fleas.

<sup>1</sup> Biraud, Y., and Deutschman, S., *League of Nations Epidem Rcp*, 1936, **15**, 99

- Zinsser, H., *Am J Hyg*, 1937, **25**, 430

<sup>3</sup> Wheatland, F. T., *Med J Australia*, 1926, **1**, 261

<sup>4</sup> Strickland, C., *Far East Assn Trop Med Trans Ninth Congress*, 1927, **2**, 517

<sup>5</sup> Lépine, P., *Compt rend Soc de biol*, 1934, **117**, 848

<sup>6</sup> Lépine, P., and Lorando, N., *Bull Soc path exot*, 1936, **29**, 285

uniformly free from the infection Sparrow,<sup>7</sup> on the other hand, recovered 2 typhus strains from the pooled brain emulsions of 10 out of 300 mice (*Mus musculus gentilis*) caught at random from different households in Tunis Brigham<sup>8</sup> also obtained a typhus strain from a field-mouse (*Peromyscus polionotus polionotus*) in the rural district of southern Alabama where cases of typhus had developed under conditions which made the rat a "highly improbable causative factor" It is clear from the above reports that hitherto the association of typhus-infected mice and mouse-fleas with the disease in man has not been conclusively demonstrated, and hence the role of mice as another reservoir of "endemic" typhus can not be considered as settled

In March, 1940, a typhus epidemic occurred in a small household in this city, affecting successively 9 out of 10 members of the family In all of them, the clinical symptomatology was characteristic, 2 were admitted into this hospital and showed, in addition, a positive Weil-Felix reaction † They admitted that they harbored lice through the greater part of the year and not infrequently encountered fleas in their clothings and beddings Rats were scarce, but the house was heavily infested with mice Four mice (*Mus wagneri*) were captured, and 12 fleas collected from these animals were identified as *Leptopsylla musculi* By inoculating separately the brains of the mice and 10 of the fleas into the peritoneal cavity of 2 guinea pigs, 2 typhus strains were recovered Each strain was passed through guinea pigs for 11 generations In most of the animals, the infection was manifested by fever (40.0-41.5°C), lasting from 2 to 9 days, after an incubation period of from 2 to 11 days In those sacrificed 10 to 14 days after the onset of fever, typhus nodules were either absent or scanty in the brains In some of the animals, the tunica vaginalis exhibited various degrees of congestion and exudation, but in none was a scrotal swelling observed After 4 and 5 passages in guinea pigs, the 2 strains were transferred to albino rats and have been carried separately from rat to rat for 10 generations to date They produced in the rats a definite febrile reaction (38.1-39.5°C) lasting in most cases from 2 to 8 days, after an incubation period of from 4 to 10 days Some of the animals killed on the 10th to the 18th day of infection gave a positive Weil-Felix reaction at titers varying from 1:10 to 1:640 The brains of rats of

<sup>7</sup> Sparrow, H, *Arch Inst Pasteur, Tunis*, 1935, **24**, 435

<sup>8</sup> Brigham, G. D., *U. S. Pub. Health Rep.*, 1937, **52**, 659

† From one of the cases, a third typhus strain was obtained from body lice in the garments, which so far does not seem to differ experimentally in any important respect from the mouse and the flea strains herein reported

the 1st and 8th generations of each series were inoculated into guinea pigs. All of the 4 animals used developed typical fever, the one inoculated intraperitoneally with the brain of a rat of the 8th generation of the flea series showed a moderately pronounced scrotal swelling, and Rickettsiae were grown from the tunica vaginalis in Matland culture and Zinsser agar tissue media.<sup>9</sup>

*Comment* In 1938, one of us (Zia),<sup>10</sup> examining over 100 mice trapped at random from different households in this city, failed to isolate typhus Rickettsia from any of these animals. The fact that the present strains were recovered from 4 mice and their fleas in a house in which an epidemic had developed naturally suggests a causal relationship between the disease in men and the infection in mice. 1 If the disease in men were historic typhus, the epidemic having started from a human carrier, it would be indeed difficult to conceive, in view of the experimental observations of Nicolle and Giroud<sup>11</sup> how the mice could have acquired the infection from man. 2 We are, therefore, inclined to think that the epidemic was of murine origin, the infection being conveyed accidentally from mice to man by mouse-fleas and thereon, independent of mice and mouse-fleas, from man to man by human body lice. Although the mouse-fleas, *L. musculi*, ordinarily do not attack man, it is not inconceivable that they may do so occasionally, when men live in close association with mice, especially if many of the mice should die in an epizootic, as during the years 1925 and 1928 in South Queensland. Moreover, as the number of mouse-fleas we examined was small, we are not certain that the mice were not infested with some other fleas which may leave their normal host more readily than *L. musculi*. In this connection it is worthy of note that a number of workers have shown that the Rickettsia of murine typhus is present in the urine of infected rats, and have suggested that the disease may be conveyed to man by ingestion of contaminated food without the mediation of fleas.<sup>13-16</sup> 3 It is, of course, possible that the initial case of the epidemic had contracted the disease from rats through rat-fleas, the mice being only secondarily involved in the course of infection among

<sup>9</sup> Zinsser, H., FitzPatrick, P., and Wei, H., *J. Exp. Med.*, 1939, 69, 179.

<sup>10</sup> Zia, S. H., unpublished observations.

<sup>11</sup> Nicolle, C., and Giroud, P., *Compt. rend. Acad. d. sc.*, 1934, 199, 1169.

<sup>12</sup> Nicolle, C., and Giroud, P., *Arch. Inst. Pasteur, Tunis*, 1935, 24, 47.

<sup>13</sup> Marcandier, A., and Pirot, R., *Bull. Soc. path. exot.*, 1933, 26, 349.

<sup>14</sup> Nicolle, C., Giroud, P., and Sparrow, H., *Arch. Inst. Pasteur, Tunis*, 1934, 23, 1.

<sup>15</sup> Le Chuiton, F., Berge, C., and Pennanéac'h, J., *Bull. Soc. path. exot.*, 1936, 29, 831.

<sup>16</sup> Le Chuiton, F., Pirot, R., Berge, C., and Pennanéac'h, J., *Bull. Acad. Med.*, 1938, 119, 175.

rats, but this does not seem very probable, since mice were greatly preponderant over rats in the house, and from the observations of Lepine and Lorando<sup>6</sup> and Sparrow,<sup>7</sup> it appears that natural transmission of typhus infection from rats to mice in the same households does not commonly occur. It must be said, however, that in general, inasmuch as rats are considerably more flea-infested than mice, and the common rat-flea, *X cheopis*, attacks man much more readily than the common mouse-flea, *L musculi*, rats are probably much more important than mice as reservoir of "endemic" typhus.

## 11850

Cultivation of *Clostridium tetani* in Unfertilized and Developing Fertilized Hens' Eggs

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The employment of fertilized egg has proven of value for the cultivation of some bacteria and a number of filterable viruses. The chorio-allantoic membrane has been mostly used as the site of infection. Since the cultivation of anaerobic microorganisms in unfertilized and developing fertilized eggs has not been recorded, the preliminary results of such a study with an obligatory anaerobe, *Cl tetani*, is herewith communicated.

**Technic** A pure toxigenic strain of *Cl tetani*, recently isolated from a patient, was used. Cultures in cooked meat tube after 48 hours' growth were used as the inoculum. Unfertilized and developing fertilized hens' eggs of different age were employed. After the position of the embryo and the air sac was identified by transillumination and marked off and the shell opposite the air sac was sterilized with tincture of iodine, a small opening was made with a carborundum disc attached to a dental drill, leaving the inner shell membrane intact, which was later cut with a small knife after resterilization with 70% alcohol and heat. A loopful of inoculum was then introduced through this opening to the center of the yolk sac, and in the case of fertilized eggs toward a point at some distance from the embryo. The shell aperture was then closed with melted hard paraffin. The eggs were then incubated at 37°C. Different batches of them were taken out and examined grossly and by

stained smears microscopically. Aerobic contaminations were excluded in every instance by absence of growth of the eggs' content on blood agar plates.

**Results** At first, cultivation was done in unfertilized eggs. Fifty-four eggs were divided into 9 groups of 6 each. One group unheated, others heated in water bath at 90°C for 0.5, 1.0, 1.5, 2.0, 3.0, 5.0, 10.0 minutes respectively, and still others fully cooked by boiling for over 15 minutes. Inoculation into the heated eggs was done after they had been cooled. One egg from each group was used for examination at 2nd, 3rd, 4th, 5th, 7th, and 14th day of incubation. From the 7th day, about  $\frac{1}{3}$  of the eggs heated for from 2-5 minutes began to show some blackening and liquefaction, at the same time a foul odor was present all of which became progressively worse. All the raw and fully cooked eggs and the remaining  $\frac{2}{3}$  of the heated eggs showed no apparent change up to 2 weeks of incubation and hardly any bacilli were found microscopically. On the other hand, numerous gram-positive bacilli with terminal spores could be seen in the liquefied and blackened eggs. The number of organisms appeared to be in direct proportion to the degree of proteolysis and blackening. The outer layer, consisting of hardened egg-white, remained intact.

It is difficult to determine the optimum length of time required for heating the eggs as the results have not been consistent. Nevertheless, the experiment did suggest the heating must be sufficient to harden the outer layer of the egg-white but must not be too long to effect a complete hardening of the egg-white.

Next, an attempt was made to grow the organisms in developing fertilized eggs of different ages. The incubation of eggs before inoculation was at 38°C. Eggs with viable embryos of 4, 6, 8, 10 and 12 days of age, were seeded with the culture broth. One egg from each group was used for examination at different periods as in the first experiment. It was found that all the eggs examined showed various degrees of proteolysis and blackening on the second day of incubation. Complete liquefaction set in from the third day on. The embryos were invariably dead. Numerous gram-positive bacilli with terminal spores were found in the contents of the eggs. The eggs with 6- to 10-day-old embryo yielded the most abundant growth. The results have been consistent and therefore dependable.

From the above experiments it can be seen that the developing fertilized eggs serve as a better medium for cultivating *Cl tetani* than the heated unfertilized eggs. A third experiment was made to in-



vestigate whether heating the fertilized eggs would enhance the growth of *Cl tetani*. Fertilized eggs with 6-7-day-old embryo were divided into 2 groups: one group unheated, the other heated in water bath at 90°C for 3 minutes, and a number of unfertilized eggs similarly heated was included for comparison. The results were somewhat unexpected. Heating of fertilized eggs retards instead of accelerates the growth of *Cl tetani*. But they still gave earlier and better growth than the heated unfertilized ones.

*Comment* In the cultivation of anaerobic microorganisms, it is well known that they are not as fastidious about their medium requirements as they are about the anaerobic environment. The growth of *Cl tetani* in some of the heated unfertilized eggs may be due to a better anaerobic condition, as heating drives off the air in the egg and the hardened layer of egg-white prevents the re-entry of air from outside.

There may be two reasons for the luxurious growth of *Cl tetani* in developing fertilized eggs. Firstly, the developing embryo consumes oxygen and thus creates a relatively better anaerobic condition, and, secondly, the presence of fresh animal tissue—the embryo—may stimulate growth. Perhaps this stimulating factor may be destroyed by heating.

The addition of animal tissue to media for the cultivation of anaerobes is not new. Von Hübner's brain medium,<sup>1</sup> and Holman's cooked meat tubes,<sup>2</sup> are notable examples. As pointed out by Holman, although the use of fresh tissue may be necessary for the growths of some of the stricter anaerobes, there is always present the disturbing possibility of contamination, not so much from without as from the animal tissues themselves.<sup>3</sup> The developing chick embryo has the advantage of being a living tissue free from contamination.

<sup>1</sup> Hübner, E. V., *Untersuchungen die pathogenen anaeroben*, Fischer, Jena, 1908.

<sup>2</sup> Holman, W. L., *J. Bact.*, 1919, 4, 149.

<sup>3</sup> a Theobald Smith, *Cent. f. Bakt.*, 1890, 7, 502, b Ford, W. W., *Trans. Assn. Am. Phys.*, 1900, 15, 389, c Wolbach, S. B., and Saiki, T., *J. Med. Res.* 1909, 21, 267.

## 11851 P

Experimental Studies upon *B. violaceus*, Isolated from a Fulminating Fatal Human Infection \*

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*Bacillus violaceus* (*Chromobacterium violaceum*) is considered a common saprophytic microorganism occurring in soil, water, sewage and contaminated food. It is generally regarded as non-pathogenic for man and lower animals.

Woolley<sup>1</sup> reported fatal infections of caribao in Manila with this microorganism which resembled hemorrhagic septicemia of cattle. He also established the pathogenicity of the isolated culture but did not find evidence of toxin-production. Recently Black and Shahan<sup>2</sup> and Soule<sup>3</sup> reported instances of human infection with *B. violaceus* which persisted for many months and finally proved fatal. Soule demonstrated pathogenicity for laboratory animals.

The culture employed in the present experiments was isolated in pure state on ordinary media from the lesions present in various organs at the necropsy of a human case. The infection arose from a wound of the foot. There ensued a marked inguinal adenitis, septicemia, as shown by a positive blood culture, and death within a week.

Studies of pathogenicity and animal protection have been performed with this culture.

*Pathogenicity Experiments* 12 rabbits, 10 guinea pigs and 12 white mice were employed. Six rabbits received intravenously 1 cc of a saline suspension containing approximately one billion microorganisms per cc. The suspension was made from the growth of *B. violaceus* on nutrient agar. All 6 animals died in approximately 24 hours. The necropsies showed no macroscopical lesions. Cultures of *B. violaceus* were recovered from the lungs, liver, spleen, and heart's blood. The microscopical study of sections revealed degenerative changes, particularly in the liver.

Six rabbits were injected intraabdominally with a broth culture of

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\* Aided by a grant from the David Trautman Schwartz Research Fund

<sup>1</sup> Woolley, P. G., *Johns Hopkins Bull.*, 1905, **16**, 89

<sup>2</sup> Black, M. E., and Shahan, J., *J. A. M. A.*, 1938, **110**, 1270

<sup>3</sup> Soule, M. H., *Abstr. Scientific Proceedings, Am. Assn. of Pathologists and Bacteriologists, Am. J. Path.*, 1939, **15**, 592

*B. violaceus* in amounts of 5 cc, 0.75 cc, 1 cc, 1.25 cc, 1.5 cc, and 2 cc. Those receiving 2 cc and 1.5 cc died in 12 hours and 24 hours respectively. *B. violaceus* was grown from heart's blood and visceral organs. The animals injected with 1.25 cc and 1 cc died in 5 days. The lungs, spleen, and liver presented light yellow foci of necrosis varying from pin-point size up to 4 mm. Microscopically the lesions presented necrosis, karyorrhexis and karyolysis with little inflammatory cellular response. These lesions conformed in gross and microscopical appearance to those present in the human case. No violet color was present but *B. violaceus* was readily recovered in pure state from all lesions and also from heart's blood.

Ten guinea pigs were injected intraabdominally, 5 receiving 0.25, 0.5, 0.75, 1.0, and 1.5 cc of saline suspension of *Bacillus violaceus* from agar slants and contained approximately one billion bacilli per cc. Five other animals received similar injections of broth culture grown for 4 days and diluted to contain approximately one billion bacilli per cc, all died within 16 hours. No discernible lesions were found although *B. violaceus* was recovered from visceral organs and heart's blood. Microscopically, degenerative changes occurred in most parenchymatous structures. Three guinea pigs receiving 0.75, 1.0, and 1.5 cc of the saline suspension died in 24 hours and presented a few minute scattered lesions in the liver. The lungs were greatly congested. Microscopically the hepatic lesions were of a granular, necrotic type showing fragmented nuclei. *B. violaceus* was recovered from the organs and heart's blood. The 2 remaining guinea pigs receiving 0.25 and 0.5 cc have survived for several weeks.

Twelve mice were injected intraabdominally, 6 with 0.1 cc and 6 with 0.5 cc of suspensions of *B. violaceus* like those employed for the guinea pigs and died within 48 hours. Those receiving the broth culture died more quickly than those injected with the saline suspensions from agar slants. No gross lesions were produced but the microscopical study revealed evidence of toxic degenerative changes particularly in the liver lobules. *B. violaceus* grew readily from the heart's blood, liver and spleen.

To ascertain if this human strain of *B. violaceus* possessed an exotoxin, cultures were grown in flasks of broth. After incubation for 24 and 96 hours the cultures were filtered through a Seitz filter at these respective periods. It was found that intraabdominal injections of as much as 2 cc of this sterile filtrate into white mice produced no ill effects. When these animals were later injected with fatal doses of *B. violaceus* they remained normal although control animals

receiving the same dosage died overnight Further tests for such protection were, therefore, made

*Animal Protection Experiments* Eight white mice were injected intraabdominally with 0.5 cc of sterile broth Seitz filtrate obtained from a 24-hour culture After 3 days, 1.5 cc of a similar filtrate from a broth culture grown for 96 hours, was likewise administered to these animals Two days later all animals received intraabdominally 0.1 cc of a virulent *B. violaceus* broth culture All animals survived and showed no evidence of illness during several weeks of observation

Of 10 control mice, 5 received intraabdominally 0.5 cc and 5 received 1.0 cc of the same culture as used for the above animals and all died overnight

Two control mice were injected with plain sterile broth in similar amounts, route and time periods as those receiving the broth culture filtrate The injection 48 hours later of 0.1 cc of the living culture produced death within 24 hours

It is concluded from these observations that this strain of *B. violaceus* isolated from an unique clinical case, is pathogenic for various laboratory animals When the inoculum permits of survival for several days, the lesions produced are similar in the gross and microscopic aspect to those of the human

It is further shown that the injection of *B. violaceus* broth culture filtrates into white mice affords protection to doses of the living microorganism that kill control animals overnight

Preliminary experiments with the serum of mice injected with sterile filtrates suggest that antitoxic factors are developed by which passive protection may be transferred to normal animals

11852

### A New Method for Regulated Vascular Obstruction

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M. B. Visscher)

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Restriction of blood flow in isolated vascular territories in living and unanesthetized animals has recently attracted attention Some workers have fitted the blood vessels which are to be constricted into a type of rigid frame which reduces the diameter of the vessels

Other investigators (Goldblatt,<sup>1</sup> Collins,<sup>2</sup> Ryland<sup>3</sup>) achieved the desired degree of ischemia by tightening loops of wire or thread around the vessel Brotchner<sup>4</sup> and Blum, Schauer and Calef<sup>5</sup> employ a device which permits gradual mechanical closure of the vessel by means of 2 solid bars which are brought together by a screw to be actuated by a stiff wire from outside the animal

The work of Mann, Herrick, Essex, and Baldes<sup>6</sup> correlated blood flow in vessels with a decrease in internal diameter brought about by local constriction It was shown that a significant diminution of flow was achieved only after reduction of the internal diameter of arteries to a small percentage of the original

The internal diameter of a vessel cannot very well be controlled quantitatively in the living animal because of the variability of the connective tissue reaction around the clamp among other reasons Once a constricting loop or a rigid frame is applied to a vessel and the wound closed, the blood flow cannot be changed without further surgical intervention Even then the percentage reduction of blood flow remains unknown until after the death of the animal, when model experiments can be performed on the flow of the blood in the constricted vessel

The method which is described here attempts to regulate flow by employing the principle of the blood pressure cuff This method should allow reversible and well defined obstruction of the vessels *in vivo*, when the pressure in the cuff can be controlled by a piece of rubber tubing which is brought out through the body wall

Balloons of suitable dimensions have been obtained by the courtesy of Mr H E Custer of the Dean Rubber Mfg Co, 16th and Iron St, North Kansas City, Mo If clamps of an irregular shape are needed it is relatively easy to make the balloons in the laboratory by dipping suitable molds into Latex\* and drying The finished clamp consists of a frame made of Lucite or Bakelite, one or two balloons which are fitted in to this frame and connecting glass and rubber tubing The assembled instrument may be sterilized by boiling The solid part has a U-shaped cross section and accommodates the vessel to be restricted in the lower part of the groove The flat

<sup>1</sup> Goldblatt, H., Lynch, Hanzal and Summerville, *J Exp Med*, 1934, **59**, 347

<sup>2</sup> Collins, D A, *Am J Physiol*, 1936, **116**, 616

<sup>3</sup> Ryland, D A, *PROC. SOC EXP BIOL AND MED*, 1938, **38**, 10

<sup>4</sup> Brotchner, R. J, *PROC SOC EXP BIOL AND MED*, 1939, **40**, 264

<sup>5</sup> Blum, L, Schauer, G, and Calef, B, *Am Heart J*, 1938, **16**, 159

<sup>6</sup> Mann, F C, Herrick, J F, Essex, H E, and Baldes, E J, *Surgery*, 1938,

balloon is then put on top of and parallel to the vessel, and the groove is closed by a tightly fitting cover. The cover is held in place by a ligature. The balloon is fixed to the cover by doubling back the free end and tying it to the frame. Stretching of the thin latex balloon is avoided by slipping its open end on a glass tube and connecting the tube to the frame of the clamp. Very sensitive vessels which should not come in contact with the solid part of the clamp may be constricted between two inflatable balloons. In order to obtain a reduction of blood flow which can be repeated consistently and quantitatively it is essential to observe a few precautions during the operation and application of the clamp.

The rubber balloon must be flaccid and without tension at atmospheric pressure. Both the artery and the balloon must fit tightly and snugly into the solid part. The balloon must fill the whole lumen of the U-groove without stretching of its walls. The artery must not be torn, constricted or kinked by the uninflated clamp. It is recommended to have available a series of clamps of varying cross-section in order to achieve the best results. Disregard of any of the above precautions may lead to results which are difficult to interpret.

Blood flow in the system composed of vessel and clamp is influenced by the following factors: pressure in the vessel, pressure in the constricting balloon, area of contact between cuff and vessel.

Of these factors the third has to be taken into consideration for the constriction of the clamp because the area of compression is determined by the length of the rigid part. If the area of contact is too small, pressures many times as high as the peak arterial pressures may fail to stop arterial flow completely. The cuff should compress the artery over a length of at least 8 mm in order to insure complete interruption of flow when the cuff pressure equals the peak arterial pressure.

The results reported below are taken from a femoral artery-femoral vein anastomosis in dogs. This arrangement eliminated peripheral resistance as a factor from our calibrations. For *in vivo* experiments the changes in peripheral resistance must be taken into account. Calibration over a range of pressure more extensive and continuous than reported can be undertaken in model circuits.

A Stomikow-stromuhr had been interposed between artery and vein. The artery was constricted as described. The systolic and diastolic pressures were recorded optically by the "spoon" method (Sedgwick and Kubicek<sup>7</sup>). The manometer needle was inserted

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<sup>7</sup> Kubicek, W. G., Sedgwick, F., and Visscher, M. B., *The Review of Scientific Instruments*, in press.

Other investigators (Goldblatt,<sup>1</sup> Collins,<sup>2</sup> Rytand<sup>3</sup>) achieved the desired degree of ischemia by tightening loops of wire or thread around the vessel. Brotchner<sup>4</sup> and Blum, Schauer and Calef<sup>5</sup> employ a device which permits gradual mechanical closure of the vessel by means of 2 solid bars which are brought together by a screw to be actuated by a stiff wire from outside the animal.

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<sup>1</sup> Goldblatt, H., Lynch, Hanzal and Summerville, *J. Exp. Med.*, 1934, **59**, 347.

<sup>2</sup> Collins, D. A., *Am. J. Physiol.*, 1936, **110**, 616.

<sup>3</sup> Rytand, D. A., *Proc. Soc. Exp. Biol. and Med.*, 1938, **38**, 10.

<sup>4</sup> Brotchner, R. J., *Proc. Soc. Exp. Biol. and Med.*, 1939, **40**, 264.

<sup>5</sup> Blum, L., Schauer, G., and Calef, B., *Am. Heart J.*, 1938, **16**, 159.

<sup>6</sup> Mann, F. C., Herrick, J. F., Essex, H. E., and Baldes, E. J., *Surgery*, 1938,

## 11853 P

## Growth of the Mammary Glands in Hypophysectomized Mice \*

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Many investigations have indicated that the hypophysis is essential for mammary growth in animals receiving estrogen (see <sup>1</sup> and <sup>2</sup> for review) The above observations, however, have not been uniform in animals of different species or, in some instances, among animals of the same species observed in different laboratories In hypophysectomized male mice the injection of large amounts of estrogens for prolonged periods<sup>3</sup> did not induce mammary growth On the other hand mammary growth followed the injections of a lipid extract of the pituitary gland and led to the assumption that the pituitary produced a mammogenic hormone <sup>4</sup>

Recent experiments have demonstrated that estrogens will induce a local growth of the breast tissue of rabbits<sup>5</sup> and monkeys<sup>6</sup> if properly applied while adjacent mammary glands in the same animal are not affected Similar experiments conducted with mice have given comparable results <sup>7</sup> Mammary growth has also occurred in young male mice receiving injections of progesterone,<sup>8</sup> several androgens and desoxycorticosterone as well as estrogens<sup>9</sup> The apparently contradictory observations listed above indicated the desirability of further studies on the estrogen-hypophyseal-mammary relationship

One-hundred-one hybrid mice from 30 to 80 days old were hypo-

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\* These investigations have been supported by the Jane Coffin Childs Memorial Fund, the Anna Fuller Fund and the Fluid Research Funds of Yale University School of Medicine

<sup>1</sup> Turner, C W, *Mammary Glands*, Chapter XI, Sex and Internal Secretions, E Allen, C H. Danforth and E A Doisy, Editors, Williams & Wilkins, Baltimore, Nelson, W O, *Physiol Rev*, 1936, **16**, 488

<sup>2</sup> Fredrikson, H., *Acta Obst et Gynec Scand*, **19**, Suppl. 1

<sup>3</sup> Gomez, E T, Turner, C W, Gardner, W U, and Hill, R T, *Proc. Soc. Exp Biol. and Med.*, 1937, **36**, 287

<sup>4</sup> Lewis, A A, and Turner, C W, *Proc. Soc. Exp Biol. and Med.*, 1938, **39**, 435, *Mo Agr Exp Sta Res Bul* 282, 1938

<sup>5</sup> Lyons, W R, and Sako, Y, *Proc. Soc. Exp Biol. and Med.*, 1940, **44**, 398

<sup>6</sup> Speert, H, *Science*, 1940, **92**, 461

<sup>7</sup> Gardner, W U, *Yale J Biol and Med*, in press

<sup>8</sup> Gardner, W U, and Hill, R T, *Proc. Soc. Exp Biol. and Med.*, 1936, **34**, 718

<sup>9</sup> Van Heuverswyn, J, Folley, S J, and Gardner, W U, *Proc. Soc. Exp Biol. and Med.*, 1939, **41**, 389



TABLE I

Cuff pressure	Diastolic pressure	Systolic pressure	Flow (in % of unobstructed 120 cc/min)
0	120	170	100
14	120	170	100
32	130	180	66
66	125	175	50
66	135	175	50
88	135	175	33
104	140	175	20
122	135	175	18
132	135	175	10
143	135	175	8
156	135	175	5
165	130	175	3
174	135	170	2
188	135	175	1
209	130	175	0
150	125	170	2
140	130	170	3
128	125	175	3
112	125	170	7
94	130	175	9
81	130	180	10
109	95	140	5
85	95	145	9
30	105	150	40
8	110	150	55
74	115	160	20
136	120	160	6
173	115	155	0
160	115	155	15
103	115	155	10
5	110	150	70

longitudinally into the artery, cardial to the constriction, with the bevel pointing towards the heart. The cuff pressure was read directly from a mercury manometer.

The reported figures indicate the gradations in flow which may be obtained with the above described device. It is believed that more satisfactory regulated and reversible restriction of blood flow can be achieved in this way than by other methods.

20 to 50 mitotic figures were counted in individual terminal buds of the proliferating ducts. The total size of the glands averaged less than those of intact animals similarly treated. The response of the glands of the incompletely operated animals was usually quite similar to that of the completely hypophysectomized except in a few instances. The very small amount of pituitary tissue remaining in some incompletely operated animals, no anterior lobe-tissue in some instances, did not greatly alter the quantitative response of the glands.

The amount of mammary growth occurring and the relative number of mice responding was smaller in the completely operated animals of the groups receiving desoxycorticosterone acetate, progesterone or estradiol dipropionate. The glands of these animals were only slightly enlarged and comparatively few dividing cells were present in the end buds of the ducts. The glands of the incompletely operated mice receiving desoxycorticosterone acetate, progesterone or estradiol dipropionate were all more extensively developed if anterior lobe tissue remained.

The complete lack of mammary growth in the mice receiving testosterone propionate + estradiol dipropionate cannot be explained at this time as both of these hormones alone induced a definite but slight mammary growth. The more extensive mammary proliferation of the mice receiving desoxycorticosterone acetate + estradiol dipropionate or progesterone + estradiol dipropionate is not considered to be only an additive effect.

*Summary* Slight growth of mammary glands of some hypophysectomized male mice was induced within 15 days by the injection of desoxycorticosterone acetate, progesterone and estradiol dipropionate. A more extensive and more rapid proliferation of the mammary ducts of hypophysectomized mice occurred when desoxycorticosterone acetate or progesterone was injected with the estradiol dipropionate.

physectomized using the parapharyngeal method described by Thomas<sup>10</sup> Testosterone propionate, desoxycorticosterone acetate or progesterone† alone or in combination with estrogens or estrogen alone were administered for periods of 12 to 15 days starting from 1 to 89 days postoperatively All hormones were dissolved in sesame oil and injected subcutaneously The mice were fed Purina Fox Chow and water The mammary glands were removed on the skins and prepared as "whole mounts" The completeness of hypophysectomy was checked by examination of serial sections of the sella turcica and surrounding tissues

The 7 groups of mice in the present experiment are listed in Table I Each of these groups was composed of smaller units of 3 to 6 animals Each of the animals of these smaller groups received the same amount of hormone while the amounts varied among the groups The range of the daily doses administered is indicated in the table

All of the substances used induced mammary growth in hypophysectomized mice except the testosterone propionate The relative number of mice showing mammary growth and the extent of mammary growth was greatest in those receiving desoxycorticosterone acetate + estradiol dipropionate, or progesterone + estradiol dipropionate The glands were markedly enlarged, and from

TABLE I.

Summary of the Treatment and Mammary Response of Completely and Partially Hypophysectomized Mice Receiving Several Hormones

Treatment hormones used	No of mice	No with complete hypophysec tomy	No with incomplete hypophysec tomy	Treatment amt daily	No of mammary responses	
					Complete hypophysec tomy	Incomplete hypophysec tomy
TP*	16	11	5	.25 to 1.25 mg	0	1
DC	10	7	3	25 mg	2	2
P	12	4	8	125 to 0.25 mg	1	3
E	19	10	9	0.5 µg to 1 µg	5	8
DC and E	18	14†	4	DC and E‡	12	3
P and E	16	12	4	P and E‡	11	4
TP and E	10	7	3	TP and E‡	0	0

\*TP—Testosterone propionate, DC—Desoxycorticosterone acetate, P—Progesterone, E—Estradiol dipropionate

†One mouse in this group had no mammary glands

‡The amounts of the various hormones used in these groups varied as indicated above.

10 Thomas, F, *Endocrinology*, 1938, 23, 99

† The testosterone propionate and progesterone were generously supplied by Dr E Schwenk of the Schering Corporation and the desoxycorticosterone acetate and estradiol dipropionate by Drs Mautner and Oppenheimer of Ciba Pharmaceutical Products

tected against both the Japanese and the St Louis viruses. Furthermore, Webster<sup>4</sup> obtained no cross protection against the Japanese virus with the serum of a monkey hyperimmunized with the St Louis virus, but with serum from a monkey hyperimmunized with the Japanese virus, mouse protection was afforded not only against the Japanese virus but against at least one minimal lethal dose of the St Louis virus. This cross protection with the Japanese serum is not emphasized by Webster, although it appears in the table of his results.

In contrast to the results of others Perdrau,<sup>6</sup> in experiments published posthumously, demonstrated neutralization of the homologous and the heterologous virus to approximately the same extent with antisera prepared in rabbits by hyperimmunization with either the virus of Japanese encephalitis or the virus of St. Louis encephalitis. These results are not in accord with the other published data.\*

In view of the conflicting evidence reported in the literature, it seems desirable to report our results obtained with the sera of mice and rabbits hyperimmunized with the viruses of St Louis and Japanese encephalitis.

*Materials and Methods. Virus.* The Nakayama strain of Japanese encephalitis virus and the Hubbard strain of St Louis encephalitis virus were employed in these experiments.

*Serum.* The serum was prepared in the following manner. Three groups of mice were immunized with the Japanese virus by repeated subcutaneous inoculations of 25 cc of dilutions of fresh infected mouse brain. Each group received 10 weekly inoculations. For the first inoculation a virus dilution of  $10^{-5}$  was employed. The strength of the virus dilution used was increased with subsequent inoculations, so that a dilution of  $10^{-2}$  was used for the final 4 inoculations. Each group of mice was bled one week after the last injection. The sera from the individual mice of each lot were pooled.

St Louis virus immune serum was prepared in mice in a similar manner. Two groups of mice were immunized. One group received 11 inoculations, and the other, 14 inoculations. A virus dilution of  $10^{-3}$  was used for the first few inoculations, and for later inoculations, a stronger dilution,  $10^{-2}$ . The sera from the mice in each group were pooled.

Immune sera were prepared in rabbits by immunizing them with

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<sup>6</sup> Perdrau, J. R., *J. Path. and Bact.*, 1940, 50, 545.

\* Webster has recently published the results of a study of the strains of virus used by Perdrau. He reached the conclusion that some of the material had been mislabeled and that Perdrau had been dealing only with strains of the St Louis virus. Webster, L. T., *Proc. Soc. Exp. Biol. and Med.*, 1940, 45, 499.

## Serological Relationship Between the Viruses of Japanese B and St Louis Encephalitis

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R. A Moore )

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St Louis, Mo*

Although many points of resemblance between the viruses of St Louis and Japanese B encephalitis have been emphasized, the evidence supporting their serological relationship is conflicting

In neutralization tests in mice, Kawamura<sup>1</sup> and his associates found no cross protection with antisera prepared in mice, rabbits and goats by immunization with the viruses of Japanese and St Louis encephalitis, nor with sera of patients convalescent of the two diseases Kasahara<sup>2</sup> and his colleagues found that the Japanese hyperimmune serum of rabbits neutralized both the Japanese virus and the St Louis virus, but the latter only to a slight degree On the other hand, the St Louis immune serum neutralized only the St Louis virus With one of the strains of St Louis virus used, this slight cross protection was apparent when the virus-serum mixtures were instilled intranasally but not when inoculated intracerebrally

Kudo<sup>3</sup> and his collaborators observed that sera of rabbits immunized with the Japanese encephalitis virus neutralized not only the homologous virus, but to some extent the St Louis virus Their tables show that protection of mice against 1 to 100 minimal lethal intracerebral doses of the St Louis virus was afforded by immune sera from each of 3 rabbits

In protection tests in mice, Webster<sup>4</sup> found no cross neutralization of the Japanese and St Louis viruses with the sera of individuals convalescent from either Japanese B encephalitis or St Louis encephalitis However, he cites an instance of cross neutralization with human sera noted by Kuttner<sup>5</sup> Sera obtained by Kuttner from two Europeans who had contracted encephalitis in China pro-

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<sup>1</sup> Kawamura, R., Kodama, M., Ito, T., Yasaki, T., and Kobayakawa, Y, *Arch Path*, 1936, **22**, 510

<sup>2</sup> Kasahara, S., Yamada, R., and Hamano, R., *Kitsato Arch Exp Med*, 1937, **14**, 22

<sup>3</sup> Kudo, M., Uruguchi, K., Matsuda, S., and Hashimoto, K., *J Immunol*, 1937, **32**, 129

<sup>4</sup> Webster, L. T., *J Exp Med*, 1938, **67**, 109

<sup>5</sup> Kuttner, A., and T'ung, T., *J Clin Inv*, 1936, **15**, 525

afforded not only against the Japanese virus, but to a slight extent against the St. Louis virus (Table III). This protection against the heterologous virus, although slight against only one to 10 minimal lethal intracerebral doses, appears to be of significance since it occurred with all 3 sera. With the Japanese immune rabbit serum, the same slight neutralization of the heterologous virus was observed (Table IV). These results are in agreement with those obtained by Kudo and by Kasahara with immune rabbit sera. They are also in agreement with the findings recorded by Webster with immune

TABLE II.

Neutralization Tests Using St. Louis Immune Rabbit Sera with the Japanese B Encephalitis Virus and with the St. Louis Encephalitis Virus

Rabbit sera	Dilution of Japanese virus added to serum				
	10-3	10-4	10-5	10-6	10-7
Normal	—	—	5, 6, 7, 8	6, 8, S, S	—
St. Louis Imm. No. 391	—	—	(1), 6, 7, 7	7, 8, S, S	—
Normal	—	—	5, 6, 6, 7	6, S, S, S	—
St. Louis Imm. No. 395	—	—	6, 7, S, S	S, S, S, S	—

	Dilution of St. Louis virus added to serum				
	10-3	10-4	10-5	10-6	10-7
Normal	—	—	4, 4, 5, 5	4, 5, 5, 6	4, 5, 6, S
St. Louis Imm. No. 391	6, S, S, S	S, S, S, S	—	—	—
Normal	—	—	3, 4, 4, 5	4, 4, 5, 5	—
St. Louis Imm. No. 395	6, 6, 9, S	S, S, S, S	—	—	—

TABLE III.

Neutralization Tests Using Japanese Immune Mouse Sera with Japanese B Encephalitis Virus and with St. Louis Encephalitis Virus

Pooled mouse sera	Dilution of St. Louis virus added to serum			
	10-3	10-4	10-5	10-6
Normal No. 386	—	4, 5, 5, 5	(2), 5, 5, 8	5, 8, S, S
Japanese Imm. No. 385	—	5, 5, 5, 5	5, 5, 8, 8	S, S, S, S
Normal No. 366	—	4, 4, 5, 5	5, 5, 7, 8	5, 6, S, S
Japanese Imm. No. 365	—	4, 5, 6, 10	5, 8, S, S	S, S, S, S
Normal No. 386	—	—	5, 5, 5, 5	5, S, S, S
Japanese Imm. No. 405	—	—	6, S, S, S	S, S, S, S

	Dilution of Japanese virus added to serum			
	10-3	10-4	10-5	10-6
Normal No. 386	—	6, 6, 6, 6	6, 6, 6, 6	12, S, S, S
Japanese Imm. No. 385	—	8, 11, S, S	S, S, S, S	S, S, S, S
Normal No. 386	—	—	5, 6, 6, 7	6, 6, 6, 6
Japanese Imm. No. 405	6, 7, 8, 9	S, S, S, S	7, S, S, S	S, S, S, S

the Japanese and the St Louis virus respectively by repeated intra-peritoneal inoculations of active virus. Approximately 5 cc of a 10% dilution of virus in broth was used for each inoculation. Two animals immunized with the Japanese virus received 17 inoculations over a period of 10 months. Sera from these 2 rabbits were pooled. Another animal received 4 biweekly inoculations. Two rabbits immunized with the St Louis virus received 20 inoculations over a period of 10 months. All rabbits were bled 2 to 3 weeks after the final inoculation.

**Neutralization Test** Brains of mice infected with the St Louis or Japanese encephalitis virus were removed aseptically and stored in the frozen state for 1 or 2 days. At the time of the test, one brain was warmed at room temperature and made into a 10% emulsion with broth of pH 7.4. After light centrifugation the supernatant fluid was used to make serial tenfold dilutions. One part of each dilution of virus used was added to 2 parts of serum, and the virus-serum mixture was incubated for 2 hours at 37°C. Swiss mice under light ether anesthesia were inoculated intracerebrally with 0.3 cc of this mixture. Four mice were used for each dilution of virus.

**Results** The results of the cross neutralization tests appear in the tables. It is apparent that no protection was afforded against the Japanese virus with either of the 2 lots of pooled St Louis immune mouse sera, although good protection was afforded against the homologous virus (Table I). The same was true of the St Louis immune rabbit sera (Table II). On the other hand, with each of the 3 lots of pooled Japanese immune mouse sera protection was

TABLE I  
Neutralization Tests Using St. Louis Immune Mouse Sera with the Japanese B Encephalitis Virus and with the St. Louis Encephalitis Virus

Pooled mouse sera	Dilution of Japanese virus added to serum			
	10-3	10-4	10-5	10-6
Normal No 366	—	4, 6, 6, 8	(1), 6, 7, 8	6, 7, 7, 8
St. Louis Imm No 342	—	(1), 6, 7, 8	4, 5, 7, 8	(1), 6, 8, 8
Normal No 386	—	(1), 5, 5, 5	5, 6, 6, 7	6, 6, 6, 6
St. Louis Imm No 406	—	5, 6, 6, 6	5, 5, 6, 6	5, 7, 7, 7
	Dilution of St. Louis virus added to serum			
	10-3	10-4	10-5	10-6
Normal No 386	—	—	5, 5, 5, 5	5, S, S, S
St. Louis Imm. No 342	—	S, S, S, S	S, S, S, S	S, S, S, S
St. Louis Imm. No 406	S, S, S, S	13, S, S, S	S, S, S, S	S, S, S, S

Numerals indicate day of death of mouse, S = survival, ( ) = accidental death.

## Effect of Fluorine on Life Span of Rachitic Rats \*

SIDNEY B. FINN† AND MORTON KRAMER‡ (Introduced by H. C. Hodge)

*From the School of Medicine and Dentistry, University of Rochester, Rochester, N. Y.*

Although considerable study has been made of the toxic effects of fluorine upon the bones and teeth, little attention has been directed to its rôle in modifying phosphorus metabolism in certain of the deficiency diseases. The effect of fluorine upon the calcification of the long bones of rachitic rats has recently been studied roentgenographically by Morgareidge and Finn<sup>1</sup>. It was found that when rats were fed 300 p.p.m. of fluorine incorporated into a rachitogenic diet (Steenbock & Black 1925)<sup>2</sup> the rickets resulting was markedly less severe as evidenced by the narrower zone of hypertrophic metaphyseal cartilage and by the increased overall density of the tibial bone. With this apparent reduction in the severity of rickets it was interesting to speculate whether the increased density of the bone might be due to formation of a more radiopaque compound with fluorine in the bone or whether there was an actual lessening of the severity of the disease.

This experiment was undertaken to show the effect of fluorine upon the life span of rats fed a rachitogenic diet and to offer additional evidence on the specific effects of fluorine when added to such a diet. Twenty-six albino rats from our own inbred stock were divided equally at weaning age (21 days) into 2 groups. Litter mates and sexes were distributed equally. The first group was fed the rachitogenic diet (Steenbock & Black 1925) consisting of yellow corn 76%, wheat gluten 20%,  $\text{CaCO}_3$  3%, and  $\text{NaCl}$  1%. The second group of rats was placed upon a similar ration plus 300 p.p.m. of dietary fluorine. This was incorporated by thoroughly mixing a saturated solution of  $\text{NH}_4\text{F}$  in 95% alcohol with the dry diet, spreading the wet mixture in thin layers on large sheets of wrapping paper and allowing the alcohol to evaporate. The amounts of inorganic phosphorus in each diet were determined by the colori-

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\* Supported by a grant from the Carnegie Corporation of N. Y.

† Now associated with the Maternity, Infancy and Child Hygiene Division, New York State Department of Health.

‡ Assistant Statistician, New York State Department of Health.

<sup>1</sup> Morgareidge, K., and Finn, S. B., *J. Nutrition*, 1940, 20, 75.

<sup>2</sup> Steenbock, H., and Black, A., *J. Biol. Chem.*, 1925, 64, 263.



TABLE IV  
Neutralization Tests Using Japanese Immune Rabbit Sera with Japanese B Encephalitis Virus and with St Louis Encephalitis Virus

Rabbit sera	Dilution of St. Louis virus added to serum				
	10-3	10-4	10-5	10-6	10-7
Normal serum	—	—	4, 4, 5, 5	4, 5, 5, 6	4, 5, 6, 8
Japanese Imm No 399 (pooled)	—	—	5, 5, 5, 6	(1), 5, 6, 8	8, 8, 8, 8
Normal serum No 267	—	5, 5, 5, 5	5, 5, 5, 5	—	—
Japanese Imm No 270	—	5, 5, 5, 10	5, 8, 8, 8	—	—

	Dilution of Japanese virus added to serum				
	10-3	10-4	10-5	10-6	10-7
Normal serum	—	—	5, 6, 7, 8	6, 8, 8, 8	—
Japanese Imm No 399 (pooled)	9, 8, 8, 8	8, 8, 8, 8	8, 8, 8, 8	—	—

serum of the monkey, although the latter investigators apparently did not attach any significance to the findings

An accumulation of evidence shows that in protection tests in mice, a definite though slight protection against the St Louis virus by Japanese immune serum prepared in a number of animal species can be demonstrated. On the other hand, no protection against the Japanese virus is afforded by St Louis immune serum.

This partial serological relationship between two viruses resembling each other in many other respects is analogous to the relationship which has been demonstrated among the 3 viruses—virus B, the virus of herpes, and that of pseudorabies.<sup>7</sup> The 3 latter possess many properties in common, and the antisera to these viruses give a slight cross neutralization of the viruses. However, with the antisera to the viruses of pseudorabies and of herpes, the partial cross neutralization is in one direction only. The immune antiherpes serum protects slightly against the virus of pseudorabies, while the anti-pseudorabies serum has no demonstrable effect against the herpes virus. It has been suggested that a generic relationship exists between these 3 viruses. It is possible that a similar group relationship exists between the 2 viruses producing the St Louis and the Japanese type of encephalitis respectively.

A definite though slight protection against the virus of St Louis encephalitis by serum of animals immunized with the Japanese virus has been demonstrated. On the other hand no protection against the virus of Japanese encephalitis has been afforded by St Louis immune serum.

<sup>7</sup> Sabin, Albert B, *Brit J Exp Path*, 1934, 15, 372

## Effect of Fluorine on Life Span of Rachitic Rats \*

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<sup>1</sup> Morgareidge, K., and Finn, S B, *J Nutrition*, 1940, 20, 75

<sup>2</sup> Steenbock, H., and Black A., *J Biol Chem*, 1925, 64, 263

metric method of Berenblum and Chain<sup>3</sup> and found to be equal, thus precluding any possible conversion of the organic phosphorus to the inorganic form by the action of the  $\text{NH}_4\text{F}$  and alcohol. Since the fluorine ration loses its efficacy on standing, this portion of the experimental diet was made up fresh every few weeks. The rats were given the diets and distilled water *ad lib* until death occurred. Mottled enamel was observed to occur on the incisors of the rats on the fluorine supplemented diet. The age at death of each of the rats was recorded.

**Findings** Table I gives the age at death (in days) of each of the rats and reveals that 10 of the rats fed the rachitogenic diet died before they reached the 68th day of life, whereas before that age no death occurred in the group of rats fed the rachitogenic diet supplemented with fluorine. By the end of the 75th day of life none of the rats fed the rachitogenic diet survived, while 9 of the rats fed the fluorine supplemented diet were still living.

The average age at death of the rats fed the rachitogenic diet was 65.23 days while for the rats fed the fluorine supplemented diet

TABLE I.  
The Age at Death (in Days) of Each of the 13 Rats Fed the Rachitogenic Diet and of Each of the 13 Rats Fed the Rachitogenic Diet Supplemented with 300 p.p.m. Fluorine

	Rachitogenic diet Age at death (in days)	Rachitogenic diet supplemented with fluorine
	59	68
	60	73
	63	74
	63	75
	64	76
	64	76
	64	76
	64	78
	66	78
	67	80
	68	80
	71	82
	75	82
Mean age at death (days)	65.23 $\pm$ 0.81	76.77 $\pm$ 0.73
Standard deviation (days)*	4.31 $\pm$ 0.57	3.90 $\pm$ 0.52

$$t = \frac{76.77 - 65.23}{1.61} = 7.16, \text{ degrees of freedom} = 24, P < 0.0001$$

$$* \text{The standard deviation is computed by the formula } s = \sqrt{\frac{\sum (x - \bar{x})^2}{N - 1}}$$

where  $\bar{x}$  is the mean of the set of observations and  $N$  is the number of observations

<sup>3</sup> Berenblum, I., and Chain, E., *Biochem J*, 1938, **32**, 295

it was 76.77 days. When the difference between the means is tested by the t-Test,<sup>4</sup> <sup>5</sup> <sup>6</sup> this difference of 11.5 days is highly significant, the probability of such a difference occurring by chance alone being less than 0.0001. The effect of fluorine on the life span of the rats thus appears to be real.

**Summary** Fluorine, when added to a rachitogenic diet, appears to increase the life span of rats.

The authors wish to acknowledge the many suggestions of Drs. H. C. Hodge, K. Morgareidge, J. F. Volkart and D. B. Ast.

11856

## Studies on Purified Tuberculin Prepared from Bacterial Bodies

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Recent attempts<sup>1-3</sup> to increase the yield of synthetic-medium tuberculin have been based primarily on increasing the growth-rate of the bacilli and on increasing the number of bacilli per unit-volume of medium. While admitting the importance of these factors it is generally agreed that further improvement along these lines is unlikely. A new approach made in this laboratory consists of increasing the production of tuberculin by the creation of an optimal condition in the medium favoring rapid cell-autolysis after maximal growth of organisms has been attained. Some of these findings have been reported.<sup>3</sup> Since the tuberculin thus obtained consists principally of bacillary proteins it seems of importance to compare the potency of purified tuberculin prepared from relatively pure bacillary proteins with that prepared from the soluble parts of the whole culture which is generally assumed to consist of metabolic products in addition to the bacillary proteins. On the other hand it

<sup>4</sup> Fischer, R. A., *Statistical Methods for Research Workers*, 5th Edition, pp 120-125, 1934, Oliver & Boyd, London.

<sup>5</sup> Tippett, L. H. C., *The Methods of Statistics*, 2nd Edition, pp 112-116, 1937, Williams & Norgate, Ltd., London.

<sup>6</sup> Yule, G. Y., and Kendall, M. G., *Introduction to the Theory of Statistics*, 11th Edition, pp 438-443, 1937, Charles Griffin & Company, Ltd., London.

<sup>§</sup> A complete discussion of this test for determining the significance of the difference between two means is given in these references:

<sup>1</sup> White, W. C., *Am Rev Tuberc*, 1934, **30**, 707.

<sup>2</sup> Wong, S. C., and Weinzirl, J., *Ibid*, 1936, **33**, 577.

<sup>3</sup> Wong, S. C., *J Bact*, 1937, **33**, 451.

appears also of interest to determine the time required for complete extraction of the active cellular protein

**Methods** Preliminary trials have shown that autolysis of organisms was more rapid in dilute alkali than in the culture medium. In the present investigation studies were made exclusively on bacterial cells. Virulent *Mycobacterium tuberculosis*, H37, was grown for 4 weeks in liter Erlenmeyer flasks containing 300 ml of a malic-acid synthetic medium<sup>2</sup>. At the end of this period the fluid was decanted from each flask after which it was replenished with half of the original volume of sterile 0.05 N NaOH. For controls sterile distilled water was added to some of the flasks. All the flasks were reincubated at 37°C and the contents thoroughly shaken each day. The state of cellular disintegration was followed by examination of smears stained by the Ziehl-Neelsen technic every other day. When a majority of the bacilli had been found to have autolyzed each flask was tested for presence of contamination by culturing the fluid on blood-agar, pH 7.6, for 48 hours. The pH of 10 flasks was adjusted to 6.0 to 6.5 with 1-N HCl before heating at 100°C for 3 hours. The preparation will be designated as tuberculin A. Ten other flasks were heated without adjustment of pH and will be known as tuberculin B. The details of the preparation of purified tuberculin will be described elsewhere. Essentially the method is similar to that of Seibert's for PPD<sup>4</sup> with the exception that glycerol and dialysis in collodion sacs were omitted. Total nitrogen was determined by the micro-Kjeldahl method. The biological activities of both preparations were compared on human subjects with a PPD preparation kindly supplied to us by Dr. Florence Seibert. Only the first strength of the Mantoux test consisting of 0.00002 mg in 0.1 ml was compared. To rule out personal factors dilutions of all the tuberculins were done by one of us and issued to the other with different labels. The key was later supplied. Readings were made 48 hours after the injection by one of us but sometimes by a nurse. Injections were made on the dorsal surface of each forearm in approximately the same position. New tuberculin-syringes were used throughout this study.

**Properties** Both preparations are light powders and very soluble in concentrations of 0.5% with the aid of few drops of dilute NaOH. The resulting solutions were pale yellow in color. The total nitrogen of tuberculin A was 13.27% and of tuberculin B, 12.96%. Incidentally it might be mentioned that the nitrogen-content of the former is the same as purified tuberculin prepared from the soluble

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<sup>4</sup> Seibert, F. B., *Am. Rev. Tuberc.*, 1934, **30**, 713

parts of whole cultures Intravenous injections of 15 mg contained in 1.5 ml into guinea pigs weighing 300-350 g produced no harmful effects Prolonged immunization of rabbits, 150 mg per animal, showed that tuberculin B was non-antigenic while tuberculin A was weakly antigenic In the latter case only complement-fixing bodies detectable in 1:5000 dilution of the antigen with serum diluted 1:5 was obtained

*Result* The yield of purified tuberculin in both cases was about 0.5 g In contrast, the yield from the pooled supernate of 20 flasks was only 0.15 g Autolysis began to appear on the 3d day of incubation and grossly this was manifested by the disappearance of granular forms On the 14th day few intact cells could be found On the other hand, stained specimens from control flasks in which distilled water alone was added showed comparatively little cellular disintegration The heated tuberculin precipitated by trichloroacetic acid in both preparations was practically salt-free, 3 to 4 washings being sufficient to render the precipitate free from chloride and sulfate ions It was found that tuberculin B which had been heated at an alkaline pH was devoid of activity This is to be expected since it is the classical procedure for hydrolyzing proteins The result of the comparative biological activities of PPD and of tuberculin A which is prepared with pH adjusted to about 6 before heating is presented in Table I From the table it is clear that tuberculin A is identical in activity to Seibert's PPD

*Comment* The above findings show 3 points of interest First, evidence is at hand which seems to identify tuberculin with tuberculo-protein, since purified tuberculin prepared either from the bacillary protein or from the soluble parts of the whole culture presents similar biological, physical, chemical, and antigenic properties Second, the method is only applicable if the pH of the crude tuberculin is adjusted

TABLE I  
Table Showing Biological Activity of Tuberculin Prepared from Acidified Lysate

Subject	Readings in mm	
	Seibert's PPD	Tuberculin A
H T C	12 × 8	12 × 12
W L M	13 × 10	14 × 11
F H	13 × 10	12 × 10
C J L	15 × 15	15 × 12
C T Y	0	0
L C L	0	0
T F C	17 × 15	13 × 12
L L L	12 × 10	17 × 13
L C L	17 × 15	15 × 12
F Y C	12 × 11	13 × 12

to below 7 previous to heating. Conversely an alkaline pH at high temperature hydrolyses tuberculin and thereby destroys its biological activity. Third, the complete disintegration of the bacilli is a logical method in further increasing the yield of tuberculin, for the number of organisms present in most cultures grown in synthetic media is large.

*Conclusion* From the results presented above it is justifiable to conclude that tuberculoprotein is suitable for the production of purified tuberculin and that a greater yield of tuberculin over a short period of time may be obtained if the organisms are treated with dilute alkali.

### 11857 P

#### Enhancing Action of Egg-Yolk on Virulence of Meningococcus for Mice

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Nungester and his coworkers<sup>1</sup> found that gastric mucin of the hog exerts a great enhancing action on the virulence of meningococcus in experimental infection of mice. This observation has been widely confirmed and extended to a number of other microorganisms of low virulence. However, as Miller<sup>2</sup> and Nungester, *et al.*,<sup>3</sup> have pointed out that different lots of mucin varied considerably in their activities and that sterilization by excessive heat might decrease their action, difficulties have been met with in our hands to secure a potent product for experimental work. Recently, in our studies on rickettsiae cultivated in the yolk-sac of the developing chick,<sup>4</sup> it was accidentally noticed that rickettsiae in yolk was more virulent than the same organisms contained in other tissues. It was thought that possibly yolk has an enhancing action on virulence similar to that of mucin. In order to test this supposition, meningococcus has been chosen for study and the results are herewith communicated.

<sup>1</sup> Nungester, W. J., Wolf, A. A., and Jourdonais, L. F., *Proc. Soc. Exp. Biol. and Med.*, 1932, **30**, 120.

<sup>2</sup> Miller, C. P., *Proc. Soc. Exp. Biol. and Med.*, 1935, **32**, 1186.

<sup>3</sup> Nungester, W. J., Jourdonais, L. F., and Wolf, A. A., *J. Inf. Dis.*, 1936, **59**, 11.

<sup>4</sup> Pang, K. H., and Zia, S. H., *Proc. Soc. Exp. Biol. and Med.*, 1940, **45**, 76.

*Materials and Method of Study* Two freshly isolated strains of meningococcus were employed for this study. Both were of low virulence when suspended in saline or Tyrode's solution. Organisms were grown on blood-agar plate for 6 to 16 hours and made up in Tyrode's solution to 500 million organisms per ml as compared with "Wellcome" opacity-tube for the standardization of bacterial vaccines. Decimal dilutions were then made also in Tyrode's solution to obtain decreasing numbers of organisms and were then mixed with 9 volumes of diluted egg-yolk immediately before inoculation.

Fresh egg-yolk was obtained aseptically and emulsified into uniform consistency by shaking vigorously with sterile glass rods. This emulsion was very sticky and its relative viscosity was found to be over 60 as determined with an Oswald's viscosimeter. Emulsions of varying degrees of viscosity were prepared by the addition of different amounts of sterile Tyrode's solution as listed in Table I.

TABLE I  
Relative Viscosities of Different Dilutions of Egg yolk.

Relative viscosity	60	52.5	43	34	25.5	19	14.5	11	8.5	7	6.4	5
Egg yolk in ml	5	4.6	4.4	4.2	4.0	3.8	3.6	3.4	3.2	3.0	2.8	2.5
Tyrode's in ml	0	0.4	0.6	0.8	1.0	1.2	1.4	1.6	1.8	2.0	2.2	2.5

Yolk-emulsions possessing relative viscosity between 11 to 19 have been found to be most satisfactory. They were effective in enhancing the virulence of the organisms and, at the same time, sufficiently dilute to be used for inoculation employing a 27-gauge needle.

*Results* Yolk-emulsion alone in 2 ml amounts of any dilutions produced no harmful result after intraabdominal injection into white mice. On the other hand, animals receiving as few as 10 meningococci suspended in the same amount of yolk-emulsions of proper viscosity showed marked prostration, ruffling of hair, discharge from eyes, and occasional convulsion and tremor. Death, preceded by coma, usually took place in 16-48 hours. Severe bacteremia could be detected by culturing a drop of blood from the tail vein either just before or soon after death of the animals. However, when the amount of egg-yolk emulsion, irrespective of its viscosity, was decreased this enhancing action on the virulence of the organisms was also greatly reduced. If 1 ml was used instead of 2 ml to suspend the organisms, at least 1000 times more organisms were required to kill mice. As the amount of yolk-emulsion was further decreased below 0.5 ml it became practically inert.

*Comment* Emulsions of egg-yolk of certain viscosity have been found to exert an enhancing action on the virulence of 2 freshly



isolated strains of meningococcus for white mice when the proper amount of yolk-emulsion was employed and the intraabdominal route was used for inoculation. As little as 10 organisms were able to kill white mice in 16-48 hours. In contrast to the varying quality of gastric mucin, egg-yolk is easily obtainable and the material has been found to be uniform in pH value and in viscosity as long as the eggs were fresh. If aseptic care is taken in handling the material, no further sterilization is required. Emulsions of the desired viscosity may be prepared according to the data given.

11858

Immunological Studies on Proteins of *Corynebacterium diphtheriae*

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Previous studies<sup>1</sup> have shown that type-specific protein derived from the Park-Williams 8 strain of *C diphtheriae* may be converted into a group-specific protein by heating at 56°C for 30 minutes. This finding suggests that diphtheric proteins in general may not be a single antigen. However, direct chemical methods, such as fractional precipitations with ammonium sulfate, have, thus far, not revealed any antigenic differences. Likewise the application of the sensitive complement-fixation reaction has proven impractical since rabbit sera immunized with whole organisms are markedly anti-complementary and diphtheric proteins alone failed to produce potent antiserum.<sup>1</sup> If it were possible to enhance the antigenic quality of the type-specific protein in rabbits by adsorbing the protein to charcoal<sup>2</sup> the usual serological methods could then be applied. The results of such a study are described below.

**Method** Proteins were prepared<sup>1</sup> from representative strains of the serological types D41 and D43 and of group  $\lambda$ .<sup>3</sup> All were serologically active, giving precipitin-titers of 1:100,000 with the

<sup>1</sup> Wong, S. C., and Tung, T., *Proc. Soc. Exp. Biol. and Med.*, 1939, **42**, 824.

<sup>2</sup> Seibert, F. B., *J. Immunol.*, 1935, **28**, 425.

<sup>3</sup> Sia, R. H. P., and Huang, C. H., *Proc. Soc. Exp. Biol. and Med.*, 1939, **41**,

specific serum only The method of preparing the charcoal for absorption was as follows 0.2 g of animal charcoal was suspended and washed with 3 changes of sterile distilled water followed by 3 of sterile physiological saline It was then suspended in about 30 ml of saline and left at 56°C for 2 hours After removing the supernate by centrifugation, 15 cc of a 1% solution of the type-specific protein were added and thoroughly mixed It was placed in a waterbath at 37°C for 2 hours with frequent agitation Then 25 ml of sterile saline were added to the mixture and it was refrigerated The subcutaneous route of injection was employed after preliminary trials had shown it to be the most effective Only rabbits whose sera did not contain natural antibodies and were not anticomplementary were used Two animals were employed for each protein Injections were made on 2 successive days followed by 5 days of rest and a total of 8 injections, totalling 16 ml, was given to each animal Animals were bled 7 days after the last injection

**Result** All the immune sera gave specific agglutinative reactions The precipitin titers varied from 1:10,000 to 1:50,000 when undiluted serum was overlaid with varying dilutions of the antigen and all but one gave reaction only with the homologous protein Type D41 immune serum, however, gave marked cross-precipitation with the group  $\alpha$  protein The reverse of this, namely, the reaction between group  $\alpha$  immune serum and type D41 protein did not occur On the other hand, when a more sensitive method such as complement-fixation was employed the sera were found to be less specific, marked cross-reactions among the various proteins and sera being found The results showing the antigenic complexity of the diphtheric proteins are presented in Table I An examination of the last column of the table shows that the diphtheric proteins contain at least 2 antigens one specific, and a common one shared apparently by all types The specific antigen is best shown with type D41 protein which gave no reaction with group  $\alpha$  serum and only a weak reaction with type D43 serum, while the common antigen is best shown by the group  $\alpha$  protein which reacts to the same degree with the homologous as well as heterologous immune sera An exception to this is found in the immune serum of type D41 which reacted strongly with the type-specific protein of type D43 This may be due to the fact that rabbits were superimmunized and therefore this serum was unsuitable for the demonstration of the type-specificity In general, however, the complement-fixation titers of all sera were higher with homologous protein than with heterologous ones It is of interest to note that serologically inactive proteins of

TABLE I.  
Antigenic Relationship of Diphtheric Proteins

Immune serum	Protein	Precipitin titer	Complement fixation titer
Type D41	T41	1 10,000	1 100,000
	G x	1 10,000	1 100,000
	T43	Negative*	1 50,000
Group x	T41	Negative*	Negative*
	G x	1 10,000	1 500,000
	T43	Negative*	1 10,000
Type D43	T41	Negative*	1 10,000
	G x	Negative*	1 50,000
	T43	1 50,000	1 1,000,000

\*Negative in 1 1000 dilution of the antigen

*C diphtheriae*\* (1 1000) can not be rendered antigenic by adsorption to charcoal. The polysaccharides of *C diphtheriae* did not give precipitin-reactions with any of the sera but did have a complement-fixing titer of 1 10,000.

*Comment* Taking the above findings in addition to those reported elsewhere<sup>1</sup> we may be justified in arriving at the following conclusions regarding the antigenic structure of diphtheric protein. The protein is composed of at least a heat-labile type-specific antigen and a heat-stable common antigen shared by all diphtheric organisms. The specific antigen being present in larger quantity masks the reaction of the other in the precipitative tests. The common antigen, however, may be manifested either in the serum of superimmunized animals by complement-fixation tests or by inactivating the protein with heat. The specific antigen is also lost when a diphtheric organism loses its virulence as was found in the case of the protein prepared from the avirulent organisms belonging to group x. Incidentally the method of adsorbing type-specific protein to charcoal may be useful in the production of type-specific serum since even undiluted serum did not cross-agglutinate.

*Conclusion* Diphtheric protein is a complex antigen consisting of at least a type-specific antigen and a group-antigen.

\* Unpublished results

## Choleretic Action and Excretion of Cinchophen in Rabbit Bile

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Since the report of Brugsch and Horsters<sup>1</sup> many investigators have definitely proved that cinchophen, or 2-phenylquinoline-4-carboxylic acid, increases the volume output of bile in dogs and man. The choleretic effect of cinchophen in the rabbit has been disputed in the literature. Stransky<sup>2</sup> reported no increase in volume output in anesthetized and unanesthetized rabbits in doses of 0.05-0.2 g. Franke<sup>3</sup> reported that small doses of atophan, up to 0.1 g, had no effect on bile flow in anesthetized rabbits, whereas larger doses, 0.15 to 0.6 g, increased the bile flow. Matsuoko<sup>4</sup> noted a choleretic effect of atophan in rabbits.

Our work was undertaken to determine the effect of cinchophen on bile flow in anesthetized rabbits, and to correlate the effect with the amount of cinchophen excreted in the bile.

**Methods** Rabbits weighing from 1.0 to 2.5 kilos were anesthetized with nembutal (sodium pentobarbital). The abdomen was opened and the common duct cannulated near its entrance into the duodenum. The cystic duct was then ligated securely. The bile was allowed to flow for about one hour until the volume had reached a constant basal level. Cinchophen (Calco), as the sodium salt, was injected into the femoral vein in doses of 50, 25 or 10 mg per kilo of body weight. The bile was then collected at half-hour intervals for 2 hours, and the volumes noted and recorded. At the end of the experiment the bile secreted after the injection of cinchophen was pooled, so that the amount of cinchophen secreted could be determined. The method devised by Bradley<sup>5</sup> was used for the determination of cinchophen in bile.

**Results** Fifty mg of cinchophen were injected slowly intravenously into 11 rabbits. The injection of cinchophen did not cause an increase in bile volume output. The average control output of bile for the 11 rabbits was 2.6 cc per half-hour, while the average output for

<sup>1</sup> Brugsch, T, and Horsters, H, *Z. Ges. Exp. Med.*, 1923, **38**, 367.

<sup>2</sup> Stransky, E, *Biochem. Z.*, 1925, **155**, 256.

<sup>3</sup> Franke, K., *Arch. Exp. Path. u. Pharm.*, 1930, **151**, 219.

<sup>4</sup> Matsuoko, Y, *Japan J. Gastr.*, 1936, **8**, 145.

<sup>5</sup> Bradley, W. B., Ivy, A. C., *Proc. Soc. Exp. Biol. and Med.*, 1940, **45**, 143.

TABLE I  
Antigenic Relationship of Diphtheric Proteins

Immune serum	Protein	Precipitin titer	Complement fixation titer
Type D41	T41	1 10,000	1 100,000
	G x	1 10,000	1 100,000
	T43	Negative*	1 50,000
Group x	T41	Negative*	Negative*
	G x	1 10,000	1 500,000
	T43	Negative*	1 10,000
Type D43	T41	Negative*	1 10,000
	G x	Negative*	1 50,000
	T43	1 50,000	1 1,000,000

\*Negative in 1 1000 dilution of the antigen.

*C diphtheriae*\* (1 1000) can not be rendered antigenic by adsorption to charcoal. The polysaccharides of *C diphtheriae* did not give precipitin-reactions with any of the sera but did have a complement-fixing titer of 1 10,000.

*Comment* Taking the above findings in addition to those reported elsewhere<sup>1</sup> we may be justified in arriving at the following conclusions regarding the antigenic structure of diphtheric protein. The protein is composed of at least a heat-labile type-specific antigen and a heat-stable common antigen shared by all diphtheric organisms. The specific antigen being present in larger quantity masks the reaction of the other in the precipitative tests. The common antigen, however, may be manifested either in the serum of superimmunized animals by complement-fixation tests or by inactivating the protein with heat. The specific antigen is also lost when a diphtheric organism loses its virulence as was found in the case of the protein prepared from the avirulent organisms belonging to group x. Incidentally the method of adsorbing type-specific protein to charcoal may be useful in the production of type-specific serum since even undiluted serum did not cross-agglutinate.

*Conclusion* Diphtheric protein is a complex antigen consisting of at least a type-specific antigen and a group-antigen.

<sup>1</sup> Unpublished results

for this non-choleretic action of cinchophen, especially since it was at variance with the results obtained in dogs. It seemed to us that cinchophen in the doses employed did not acutely damage the liver, because sodium dehydrocholate injected after the administration of cinchophen always caused a marked increase in volume output. A study of the literature revealed that, generally, rabbits are less susceptible to cinchophen poisoning than other mammals. Myers and Goodman<sup>6</sup> reported that 300 mg of cinchophen administered to rabbits for 45 days caused only minor cellular changes in the liver. Hanzlik and Lehman<sup>7</sup> found no change in the rose bengal liver function test after feeding 18 rabbits from 0.3 to 1.6 g of cinchophen per kilo over a period of 7 to 89 days. They also found that cinchophen did not increase the sensitivity of the liver to chloroform or phosphorus poisoning. Schwartz and Simonds<sup>8</sup> were unable to obtain peptic ulcers in rabbits fed 220 to 550 mg of cinchophen per kilo per day for 76 to 99 days.

From our results on the recovery of cinchophen in the bile, it appears that the absence of choleresis in rabbits may be associated with the observation that only very small amounts of cinchophen are excreted in the bile. We have found, in previous investigations<sup>9, 10</sup> that the choleresis obtained from the administration of various bile salt preparations was associated with the presence of appreciable amounts of the bile acid in the excreted bile. In the rabbit the liver is not concerned with the excretion of cinchophen as in the dog. Since cinchophen is not excreted in significant amounts in the bile of the rabbit, it would exert no osmotic effect on the bile and extra water would not be excreted in the bile. The failure of the rabbit's liver to excrete cinchophen is the only available explanation of the fact that the drug fails to cause choleresis in the rabbit.

*Summary and Conclusions* The chlorethic action of various doses of cinchophen was studied in a relatively large number of anesthetized rabbits. Cinchophen in doses ranging from 10 to 50 mg per kilo had no appreciable effect on the volume output of bile. It was also found that, on the average, only 3 mg of cinchophen per rabbit was recovered in the bile over a period of 2 hours. Since it

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<sup>6</sup> Myers, H. B., and Goodman, L., *Arch. Int. Med.*, 1932, **40**, 946.

<sup>7</sup> Hanzlik, P. J., and Lehman, A. J., *Arch. Int. Med.*, 1933, **52**, 471.

<sup>8</sup> Schwartz, S. O., and Simonds, J. P., *Proc. Soc. Exp. Biol. and Med.*, 1935, **32**, 1183.

<sup>9</sup> Berman, A. L., Snapp, E., Ivy, A. C., Atkinson, A. J., and Hough, V. H., *Am. J. Dig. Dis.*, 1940, **7**, 333.

<sup>10</sup> Berman, A. L., Snapp, E., Ivy, A. C., Hough, V. H., and Atkinson, A. J., *Am. J. Physiol.*, in press.

the first half-hour after the injection of cinchophen was 24 cc. During the remainder of the test period, the output usually showed a gradual decrease, or showed normal fluctuations. A normal variation of  $\pm 17\%$  in volume output was noted in 5 control rabbits which received no cinchophen. The injection of 50 mg per kilo of sodium dehydrocholate 2 hours after the cinchophen provoked a brisk choleresis.

It was thought that possibly a dose of 50 mg per kilo was too large for rabbits. Therefore, in subsequent experiments 25 and 10 mg of cinchophen per kilo were injected. In 5 rabbits, 25 mg per kilo gave no choleretic response. Similar results were obtained with 10 mg of cinchophen per kilo in 3 rabbits.

It was quite obvious that, under the conditions of our experiments, cinchophen in large or small doses manifested no choleretic property in rabbits. Bradley<sup>5</sup> in his studies on anesthetized and unanesthetized biliary fistula dogs found that the increase in bile volume occurring with the administration of 50 mg of cinchophen per kilo was associated with the recovery in the bile of approximately 20 to 78% of the administered cinchophen. Thinking that this fact, confirmed by us during this study, may be concerned in the mechanism of cinchophen choleresis, the bile obtained during the experiment was analyzed for cinchophen content.

The total amount of cinchophen excreted by 11 anesthetized rabbits when 50 mg per kilo was administered was 32.7 mg or an average of approximately 3 mg per rabbit during the collection period of 2 hours. In anesthetized dogs, Bradley<sup>5</sup> recovered 51 mg of cinchophen per dog during the same period of time. When the smaller doses were administered correspondingly smaller quantities of cinchophen were recovered in the bile. No cinchophen was found in the control bile, nor in the bile obtained after the injection of sodium dehydrocholate (Decholin-Sodium) which is an excellent choleretic in rabbits according to our observations on 12 rabbits. When small amounts of cinchophen were added to bile approximately 100% recovery was obtained. It is remotely possible that the liver of the rabbit is more sensitive to sodium pentobarbital anesthesia than the liver of the dog, even though the pentobarbitalized rabbit's liver secreted briskly in response to sodium dehydrocholate. To test this possibility 50 mg of sodium cinchophen per kilo was given slowly intravenously to 3 unanesthetized (chronic) biliary fistula rabbits. A choleresis did not occur.

*Discussion* From our results it can readily be concluded that different doses of cinchophen had no appreciable effect on the volume output of bile in rabbits. Our next problem was to find the reason

Preliminary studies showed that relatively great centrifugal forces (4,000 rpm at 15 cm) were necessary to displace the plastids in the cortical plasmagel whereas to displace the granules in the mobile plasmasol relatively small centrifugal forces (1,000 rpm at 15 cm) were required. As can be seen from the tables these cortical plastids (mostly chloroplastids) apparently were inactive as ion accumulation centers, and only served to dilute the granule fraction in general. However, when centrifugation was sufficient the cytoplasm became absolutely clear showing that all granules were sedimented.

The data obtained were quite reproducible and the maximum error did not exceed  $\pm 5\%$ . Tables I and II show the change  $K^+$  or  $HP^*O_4^-$  ion concentrations in the granuloplasm after various periods of immersion of the cell in 0.01M  $K^+Cl$  or  $Na_2HP^*O_4^-$  in pond water at pH 8.2 temperature  $15 \pm 0.1^\circ C$ . Control cells, those which had not been centrifuged, were immersed in radioactive salts and then cut in half and each half measured for radioactivity. This showed that these halves each had the same ion radioactive concentration.

The times of sampling were chosen to correspond with various phases of the ion penetration process, namely 25 hr corresponding to a maximum of induced accumulation (cf Brooks<sup>1</sup>), 5 hr corresponding to a minimum of ion concentration, and 4-8 hr cor-

TABLE I  
Granule Accumulation Ratios for Various Times of Immersion of Cells in Radioactive Solutions

Immersive Solutions			
Ratio	K* Ion Concentration in Granular Protoplasm		
	K* Ion Concentration in Hyaline Protoplasm		
	Low centrifugal force	High centrifugal force	Time of immersion, hr
	0.30	0.20	0.25
	0.64	0.55	0.50
	1.10	0.60	1.00
	2.20	0.75	4.00

TABLE II

TABLE 12		
Ratio	HP*O <sub>4</sub> <sup>-</sup> Concentration in Granular Protoplast	
	HP*O <sub>4</sub> <sup>-</sup> Concentration in Hyaline Protoplast (Low centrifugal force)	Time of immersion hr
	0.80	0.25
	1.48	0.50
	2.80	1.00
	4.44	2.00
	10.55	8.00

<sup>1</sup> Brooks, S. C. *J. Cell Comp. Physiol.*, 1939, 14, 4.



was found that the absence of cinchophen choleresis in the rabbit was not due to an acute intoxication of the liver, because after cinchophen sodium dehydrocholate caused a brisk choleresis, it is tentatively concluded that cinchophen does not cause choleresis in the rabbit because it is not excreted in the bile

11860

### Radioactive Ion Distribution in Protoplasmic Granules

LORIN J MULLINS (Introduced by S C Brooks )

*From the Department of Zoology, University of California, Berkeley*

The *Nitella* cell has an extensively granulated protoplasm, measurements on frozen sections giving up to 15% of the total volume of the protoplasm as being made up of nuclei, plastids, mitochondria and non-ergastic bodies. It seems important to inquire whether these granules play a significant rôle in ion transfers, and indeed, whether these bodies might not be the main seat of ion accumulation.

To determine to what extent the granuloplasm took up ions single internodal coenocytes 5-10 cm in length of *Nitella coronata* were prepared by destroying alternate cells in the chain. Care was taken to preserve a short extension of the cellulose wall (about 5 mm long) from the adjacent cell. Surgical thread could then be tied around this cellulose projection and the cell left for 24 hours in pond water in the cold room (15°C) under conditions of intermittent light which simulated sunlight. After this treatment the cell was immersed in radioactive solutions for the desired time and then placed in a centrifuge tube, the thread tied about a cork in the tube and its length adjusted so that the cell was held clear of the sides and bottom of the tube. Centrifugal force sufficient to displace the granules in the cell was applied without any other observable damage. Control centrifuged cells kept under the previous conditions would show a gradual redistribution, reaching substantial completion in about 60 hours, of the stratified granules. These cells lived as long as untreated cells. After stratification had been obtained the experimental cells were bisected into centripetal and centrifugal halves, or fractions, and the activity of each half of the protoplasm measured under a Geiger-Muller counting tube. The only apparent error due to mixing was in the sap, which almost certainly became mixed during the sampling.

it possible to apply the photoelectric principle to measuring the low intensities of color in the cholesterol micromethod of Schoenheimer and Sperry<sup>1</sup> and have allowed the development of a technic for determining bilirubin in serum when the sample is only 0.1 cc. The sensitivity is great enough to make obligatory the use of color filters of narrow spectral transparency. Since no basically new features are involved in the electrical or optical systems, the instrument is adequately described by the details in Fig. 1. The light source is a 15-candle-power automobile bulb. The ratchets, not shown in the diagram, for centering the position of the lamp must be capable of fine lateral and horizontal adjustments in order that the beam shall traverse the absorption cell and fall on the dispersing lens, M. Energy for

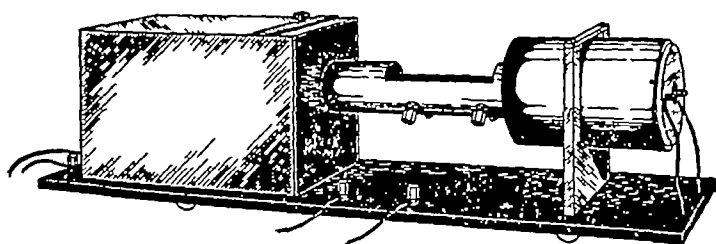
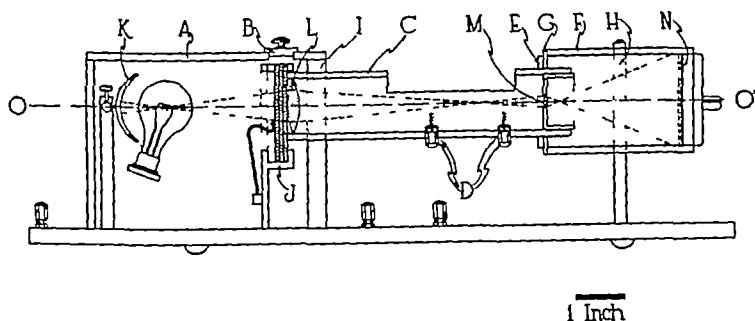


FIG 1

Diagrammatic section through the photoelectric photometer and three dimensional sketch of the instrument. The labeled parts of the diagram refer to A, housing for the light compartment, B, lid over window giving access to the color filters, C, brass tube forming the cell compartment, D, bolts for supporting the absorption cell, E, lock nut for fixing position of the cell compartment, F, compartment for the photoelement, G, end plate of the previous compartment, H, brass support, I, Bakelite support, J, compartment for the color filters, K, spherical mirror (diameter 1.75", radius of curvature 1 1/4"), L, converging lens (diameter 30 mm, focal length 37 mm), M, dispersing lens (diameter 6 mm, focal length 64 mm), N, photoelement (Lange, type S 50 or Weston photronic, type 2), OO', optical center of the instrument.

<sup>1</sup> Schoenheimer, R., and Sperry, W. M., *J. Biol. Chem.*, 1934, 106, 745; Sperry, W. M., *Am. J. Clin. Path.*, 1938, 8, 91.

responding to true primary accumulation of ions by the cell. It appears that as the accumulation of ions progresses, the concentration of these ions takes place in the granules of the cell. An objection to this technic may be that the whole protoplasm is centrifuged to the bottom of the cell under the conditions of the experiment. This, however, does not seem likely in view of the relatively strong turgor of the cells and the low centrifugal forces applied. A general discussion of ion permeability phenomena will be found in Mullins<sup>2</sup> so that only a brief explanation of the data will be given here.

Since a large concentration of ions distributed throughout the hyaline protoplasm might alter markedly the colloidal nature of the protoplasm by causing excessive solation of the colloid, it seems reasonable that there must be some stations in the cell where ions are accumulated and rendered ineffective. Apparently the vacuole of the *Nitella* cell functions in this manner, and the data here given would indicate that the granules also supplement the vacuole in this process.

11861

### A Micro Photoelectric Photometer

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Photoelectric analytical procedures depend for accuracy upon a depth of color in solution sufficient to produce satisfactory deflection of a galvanometer or other recording device. Ideally the volumes of reagents and sample are adjusted to yield 20-80% absorption of the incident light. Since the necessary intensity of color varies with the length of the path of the light beam in the solution, the dimensions of the absorption cell are limiting factors in adapting procedures to microanalysis. Present instruments on the market do not permit an absorption path longer than 20 mm when the volume of solution is limited to 1 cc.

An instrument in use at the Babies Hospital during the past 2½ years demonstrates the feasibility of constructing apparatus in which the light beam traverses a path of 50 mm through a tube-cell of narrow bore and only 1 cc capacity. These specifications have made

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<sup>2</sup> Mullins, L. J., *Ionic Equilibria in Protoplasm*, in press, 1940

color intensity was determined for the German silver cell by making a long series of readings over a number of days on two standard intensities of color. The colored solutions were composed of pure *carotene dissolved in chloroform and diluted with alcohol*, the readings were made with a blue filter. With the more dilute solution light transmission was about 70%, concentrations calculated at this level showed a standard error of  $\pm 0.65\%$ . With the more concentrated solution light transmission was approximately 25%, concentrations computed from these data disclosed a standard error of  $\pm 0.24\%$ .

The author expresses his indebtedness to Mr Kern F Larkin, who solved most of the constructional problems and to Mr Fred Rosebury, who assisted in this work.

11862 P

### Differentiation of Influenza A and Influenza B by the Complement-Fixation Reaction \*

THOMAS FRANCIS, JR

*From the Department of Bacteriology, New York University College of Medicine*

A recent report<sup>1</sup> has described the isolation of a new serological type (B) of virus from epidemic influenza. By means of the neutralization test with sera from convalescent animals and human individuals, it was shown that this virus was readily differentiated from virus of the type (A) previously identified. In the process of adaptation of the new virus to mice, attempts were made to ascertain whether serological differentiation could also be obtained with the complement-fixation reaction. While the earlier results were indefinite, these efforts have been renewed since the virus has increased in virulence for mice.

Antigens were prepared from lungs of mice infected with the PR8 strain of the virus of Influenza A<sup>2</sup> and also from mice infected with the Lee strain of Influenza B virus. Two percent suspensions of the infected lungs in physiological salt solution, after clarification by

\* This study was conducted under a grant from the International Health Division of the Rockefeller Foundation.

<sup>1</sup> Francis, T., Jr. *Science* 1940, 92, 405

<sup>2</sup> Horsfall, F. L., Jr., Lennette, E. H., Rickard, E. R., Andrewes, C. H., Smith, W., and Stuart Harris, C. H. *Lancet*, 1940, 2, 413

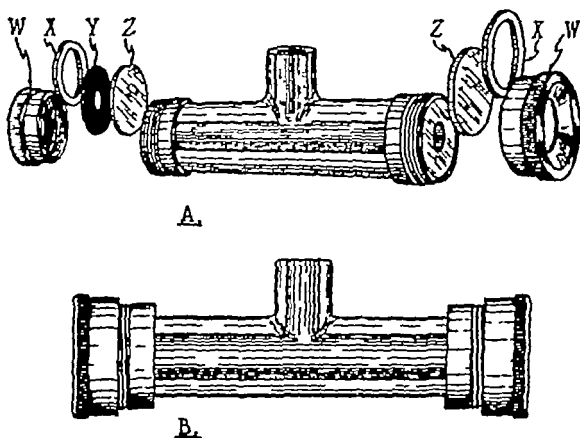


FIG 2

The absorption cell of 1 cc capacity, A indicates the constituent parts of the cell and B, the assembled cell. In addition to the main body of the cell, the parts include two metal caps, W, two rubber washers, X, a single metal diaphragm, Y, two glass windows, Z

the lamp is furnished by a 6-volt storage battery in series with a sliding-contact rheostat of 0.83 ohm resistance. The photocurrent is measured with a Lange multiflex galvanometer (maximum sensitivity,  $4 \times 10^{-9}$  amperes per mm of scale, maximum internal resistance, 1000 ohms)

The absorption cell, Fig 2, can be purchased as part of the accessory equipment of the Zeiss Pulfrich photometer. For non-corrosive solutions we use a cell of similar dimensions constructed of German silver and encased in an insulating barrel of plastic. The fidelity of readings is somewhat greater with the metal than with the glass cell, apparently because equilibration of temperature is attained more rapidly. The analytical methods examined so far have utilized organic solvents for the development of color, with such solutions there has been no difficulty in rinsing and filling the narrow-bore cell. In operation the cell is filled with a blank solution of the reagents used in the method, placed in the instrument and rotated to a fixed and reproducible position. Galvanometer sensitivity is adjusted to yield full deflection of 100 scale divisions. Because the cell acts as a condensing lens, its removal brings about a fall in the galvanometer deflection. This "air-setting" is noted and maintained throughout subsequent readings by adjustment of the galvanometer rheostat. The percentage light transmission of the unknown solutions can then be read directly as units of deflection on the scale.

For the purpose of this report the reduplicability of measures of

influenza in 1940 showed a sharp rise in antibodies to the Type B antigen but not to the antigen of Type A. In the case of patient Sq, no rise in complement-fixing antibodies was detected.

These observations, while limited in number, serve to emphasize the antigenic differences between the 2 viruses and to indicate that the complement-fixation reaction can be employed for the differential diagnosis of epidemic influenza due to virus of Types A and B.

### 11863

#### The Diurnal Levels of Blood Leukocytes in the Normal Rabbit.

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Louisiana State University*

Extensive studies on the blood of normal rabbits,\* which were made for another purpose,<sup>1, 2</sup> seemed to offer an unique opportunity to determine whether a definite diurnal cycle in the leukocyte level actually exists in this species. The animal material used had been acclimated to the laboratory for months or years before the counts were made. The exact age of the animals, as well as their breed and physical condition, was known in most instances. Counts were not made oftener than 3 times a week, and usually only once a week. The exact time of day at which the blood was taken had been recorded for each determination. Finally, the material was sufficiently extensive to make statistical analysis possible.

*Materials and Methods* Although blood counts on normal rabbits were available for all months of the year, the November and December counts were selected for analysis because in these months the largest samples were available for each hour of the day from 9 a m to 5 p m. The variation in the formula of the blood between the 2 months was not significant.

The animal material consisted of 204 young adult male rabbits examined in the months of November and December in the years

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\* These studies were done at the Rockefeller Institute for Medical Research, in the laboratory of Dr. Wade H. Brown. They were carried out by the author, in coöperation with Drs. Louise Pearce, Paul D. Rosahn, and C. K. Hu. The technical assistance of Mr. Leshe Kish was invaluable.

1 Pearce, L., and Casey, A. E., *J. Exp. Med.*, 1930, 51, 83.

2 Casey, A. E., Rosahn, P. D., Hu, C. K., and Pearce, L., *J. Exp. Med.*, 1936, 64, 453.

centrifugation, were used in the tests. Two units of complement were employed. Serial two-fold dilutions of serum were made and 0.1 cc of each dilution was mixed with 0.1 cc volumes of complement, antigen and saline. After incubation for 60 minutes at 37°C, 0.2 cc of sensitized cells were added. The tubes were again incubated in the waterbath for 45 minutes when readings were made. A complete set of controls was included in each test.

Acute and convalescent sera obtained from patients infected with Type A virus in 1938-39 and from others shown to have been infected in 1940 with Type B virus were tested for their capacity to fix complement in the presence of each antigen. Representative results are presented in Table I, where the titer of neutralizing antibodies in each serum against 100 M L D of the 2 types of virus is also recorded.

It is seen that the convalescent sera of patients who had suffered from epidemic influenza in 1938-39 showed an increase in complement-fixing antibodies to the PR8 strain of Influenza A virus but not to the Lee strain of Influenza B virus. On the other hand, the convalescent sera from 4 of the patients suffering from epidemic

TABLE I  
Complement Fixation with Sera of Patients and Influenza Virus, Types A and B

Type of Infection	Pt	Serum	Type A (PR8) antigen							Type B (Lee) antigen							Neutralization titer	
			Serum dilution 1							Serum dilution 1							Type A	Type B
			2	4	8	16	32	C		2	4	8	16	32	C			
Influenza A 1938-1939	V	A*	4	2	0	0	0	0		4	1	0			0		6	0
		C	4	4	4	4	1	0		4	1	0			0		240+	0
		J	1	0	0	0	0	0		3	0	0			0		4	6
	A	C	4	3	1	0		0		1	0	0			0		140	6
		A	4	0	0	0	0	0		3	0	0			0		30	0
		C	4	4	4	2	0	0		1	0	0			0		240	6
	L	A	0	0	0			0		0	0	0			0		120	6
		C	4	3	0			0		0	0	0			0		480	6
	Ac	A	0	0	0			0		0	0	0			0		30	8
		C	4	2	0			0		0	0	0			0		120	15
Influenza B 1940	M	A	0	0	0			0		0	0	0	0	0	0		17	0
		C	0	0	0			0		4	4	3	2	0	0		17	60
	T	A	0	0	0			0		0	0	0	0	0	0		10	12
		C	0	0	0			0		4	4	4	3	0	0		16	240
	C	A	2	1	0			0		3	1	0	0	0	0		240+	6
		C	3	1	0			0		4	4	4	4	4	0		240+	120
	L	A	4	4	1	0		0		2	1	0	0	0	0		240+	0
		C	4	4	1	0		0		4	4	4	4	1	0		240+	200
	S	A	0	0	0			0		0	0	0			0		240+	0
		C	2	0	0			0		0	0	0			0		240+	140

0 = no fixation of complement.

1, 2, 3, 4 = increasing degrees of fixation

\*A = acute, O = convalescent

TABLE I.  
Hourly Means of Total White Blood Cell Counts in Normal Male Rabbits

Hour	9 00 9 59	10 00 10 59	11 00 11 59	12 00* 1 59	2 00 2 59	3 00* 4 59	9 12 a.m.	12 5 p.m.
Counts	178	226	193	112	124	130	597	366
Animals	80	103	115	73	64	46	190	136
Mean	7709	7931	8033	8061	7945	7738	7891	7914
$\sigma_m$	$\pm 220$	$\pm 195$	$\pm 211$	$\pm 276$	$\pm 264$	$\pm 255$	$\pm 119$	$\pm 152$

\*The data from 12 noon to 1 59 p.m. and from 3 00 to 4 59 p.m. were combined to give samples more comparable with those for the other hourly periods

No single individual hourly mean differed from any other. Such variations as occurred could be explained on the basis of random sampling. There is therefore no evidence of a significant orderly variation in the total white blood cells of the rabbit in the hours between 9 a.m. and 5 p.m.

There is also no evidence in the rabbit that a digestive leukocytosis occurs between the hours of 9 a.m. and 5 p.m., as no statistically significant change was found in the group permitted free access to food and water.

*Discussion.* It has been observed that the lowest of repeated white blood cell counts in man are obtained early in the morning and the highest late in the afternoon.<sup>2-14</sup> The afternoon level is high even when food intake, muscular effort and emotional stress are controlled.<sup>6, 7, 10-14</sup> The controlled studies, however, are based on 12-50 counts made on the same day in the same persons, and the possible effect of the repetition cannot be discounted. It has been pointed out that successive painful punctures cause a successive rise in the leukocyte count,<sup>12</sup> and an analysis of the average hourly counts in the controlled series available on healthy individuals<sup>10-14</sup> reveals a

<sup>3</sup> Reinert, E., *Die Zählung der Blutkörperchen und ihre Bedeutung für die Diagnose und Therapie*, F. C. W. Vogel, Leipzig, 1891.

<sup>4</sup> Rieder, H., *Beiträge zur Kenntnis der Leukocytosis und verwandter Zustände des Blutes*, F. C. W. Vogel, Leipzig, 1892.

<sup>5</sup> Gregor, K., *Arch. f. Verdauungskrankh.*, 1898, **3**, 387.

<sup>6</sup> Japha, A., *Jahr f. Kinderh.*, 1900, **52**, 242.

<sup>7</sup> Japha, A., *Deutsch. Aertz. Zeitung*, 1901, **3**, 145.

<sup>8</sup> Türk, W., *Vorlesungen über klinische Hämatologie*, W. Braumüller, Vienna, 1912.

<sup>9</sup> Mauriac, P., and Cabonat, P., *Paris Méd.*, 1921, **11**, 407.

<sup>10</sup> Sabin, F. R., Cunningham, R. S., Doan, C. A., and Kindwahl, J. A., *Bull. Johns Hopkins Hosp.*, 1925, **37**, 14.

<sup>11</sup> Shaw, A. F. B., *J. Path. and Bact.*, 1927, **30**, 1.

<sup>12</sup> Garrey, W. E., *Proc. Staff Meet., Mayo Clin.*, 1929, **4**, 157.

<sup>13</sup> Smith, C., and McDowell, A. M., *Arch. Int. Med.*, 1929, **43**, 68.

<sup>14</sup> Kenyon, F., and Macy, I. G., *Human Biol.*, 1938, **10**, 511.



1927-1933 inclusive The age range was from 4 to 15 months and the average age was 8 months All the animals were caged separately The rabbits were usually alternated from week to week when repeated counts were made, so that counts are available for different times of the day on the same animal In all, 963 counts (an average of about 5 per animal) were made, distributed as follows

178 counts on 80 rabbits between 9 and 9 59 a m									
226	"	"	103	"	"	10	"	10 59	"
193	"	"	115	"	"	11	"	11 59	"
30	"	"	19	"	"	12	noon and 12 59	p m	
82	"	"	60	"	"	1	and 1 59	p m.	
124	"	"	64	"	"	2	"	2 59	"
85	"	"	35	"	"	3	"	3 59	"
45	"	"	17	"	"	4	"	4 59	"

In all, 597 determinations on 190 of the 204 rabbits were made between 9 a m and 12 noon, and 366 determinations on 136 of the 204 rabbits were made between 12 noon and 5 p m

The blood was collected in U S standardized Trenner pipettes Acetic acid was employed as a diluent The counts were made in a Levy-Hausser counting chamber The counting technic was standardized, so that the average coefficient of variation due to technical error in a given total white count was 9%

The material was analyzed in 2 periods, 1927-1929 inclusive, and 1930-1933 inclusive In the first period the counts were made before the animals had been fed or permitted access to water, and only one white cell pipette was filled from each rabbit on a given day In this period the material consisted of generally brown-gray hybrids purchased from dealers In the second period the animals were permitted free access to water and food at all times From 2 to 4 pipettes were filled at a single sitting and the results were averaged In this period the material consisted for the most part of animals bred in the laboratory and of known genetic stock Analysis on this basis afforded an opportunity for comparing results in animals of unknown hybrid stock without free access to food and water with results in animals of known genetic origin which had usually been in the laboratory for several generations and which were permitted free access to food and water

*Results* The mean values of the white blood cell counts for each hour of the day between 9 a m and 5 p m are presented in tabular form, together with the standard error of the mean The variance between the means of the hours was not significantly different from the variance within the means The white blood cells averaged 7,891 for the morning and 7,914 for the afternoon period, a difference of  $23 \pm 194$  white blood cells (not significant)

# Thermo-Regulatory Function of Rat Scrotum I Normal Development and Effect of Castration \*

FREDERICK N ANDREWS † (Introduced by Fred F McKenzie)

*From Missouri Agricultural Experiment Station and the United States Department of Agriculture cooperating*

It has been shown that the scrotum of certain mammals has a thermo-regulatory function,<sup>1 2</sup> and that if the testes are experimentally placed within the abdominal cavity the germinal epithelium undergoes marked degeneration.<sup>3 4</sup> In the ram, bull and boar the scrotum acquires the ability to contract and relax to temperature change as the testes begin the production of spermatozoa, and loses this ability following castration.<sup>5</sup> Further studies on the ram indicated that this function was apparently dependent upon the male hormone. Although the relations of temperature and contractility of the rat scrotum have not been investigated, Hamilton<sup>6</sup> reports that the rat scrotum depends upon male hormone for its development and maintenance and Wells<sup>7</sup> has demonstrated the dependence of the scrotal sac upon male hormone in the ground squirrel.

**Procedure** The results of studies of the thermo-regulatory function of 7 normal and 6 castrate albino rat scrota are presented (age 9 weeks to maturity, Table I). Castration was performed at 5 weeks of age through a medial, abdominal incision. The contractility of the scrotum was measured by passing a thread attached to a recording lever through the skin on the ventral surface of the scrotum midway between the scrotal tip and the body, the rats were then anesthetized with nembutal and ether, securely fastened to a heavy animal board, and submerged, with the exception of the head, in a water bath. The temperature of the bath was decreased

\*Contribution from the Department of Animal Husbandry, Missouri Agricultural Experiment Station, Journal Series No 700

† The author wishes to express his appreciation to Dr L J Wells of the Department of Anatomy, University of Minnesota, for his interest and advice in this study

<sup>1</sup> Moore, C R, and Quick, W J, *Am J Physiol*, 1924, **68**, 70

<sup>2</sup> Phillips, R W, and McKenzie, F F, *Mo Agr Exp Sta Res Bul* 217, 1934.

<sup>3</sup> Griffiths, J, *J Anat and Physiol*, 1893, **27**, 482

<sup>4</sup> Moore, C R, *Anat Rec*, 1922, **24**, 383

<sup>5</sup> Phillips, R W, and Andrews, F N, *Mass Agr Exp Sta Bul* 331, 1936

<sup>6</sup> Hamilton, J B, *Proc Soc Exp Biol and Med*, 1936, **35**, 386

<sup>7</sup> Wells, L J, *Proc Soc Exp Biol and Med*, 1937, **36**, 625

steady rise in the mean count until the series were variously discontinued at 4, 5, 7 and 8 p m. The total rise for the 4 series in question averaged  $2,330 \pm 292$  white blood cells (statistically significant). Most of the studies in which variations and rhythms were reported are based on an inadequate number of instances, and no statistical consideration of possible technical variations seems to have been undertaken.

The study herewith reported seems free from these disadvantages. The animal material was accustomed to existence in the laboratory and most of it was also accustomed to the taking of blood for examination. These circumstances perhaps lessened variations which might have been caused by bringing in animals to lead an unaccustomed laboratory existence and making the blood counts while they were in a state of digestive, environmental and emotional disturbance. In addition, only one count was made on a given animal during any given 3-day period. The data therefore seemed to rule out a possible diurnal variation in the total leukocyte count of the rabbit under controlled laboratory conditions.

When the material was examined with the idea of determining a possible bias for certain breeds counted at certain hours of the day, it was found that the breeds had been so varied that no bias of this kind was evident.

*Summary and Conclusions* Nine hundred sixty-three total white blood cell counts made on 204 normal and apparently healthy young adult male rabbits between 9 a m and 5 p m revealed no statistically significant variation in the hourly means. The blood cell level, apart from sampling variations, was the same for each hour of the day. There was also no evidence of a digestive leukocytosis in the rabbit.

The mean value for 597 leukocyte counts on 190 of the 204 rabbits between 9 a m and 12 noon was 7,891, as compared with 7,914 for 366 counts on 136 of the 204 rabbits between 12 noon and 5 p m. The difference ( $23 \pm 194$ ) was not significant.

These studies are significant for several reasons. The investigation covered a wide variety of breeds and hybrids. The animals were accustomed to laboratory existence and hemocytologic technic before the counts were made. Feeding conditions were varied during the study. Only one examination was made on a given animal on a given day, and usually not more than one or two counts were made on each animal per week. Finally, the material was analyzed by acceptable biometric procedures and represents by far the most extensive study of the sort which has been made.

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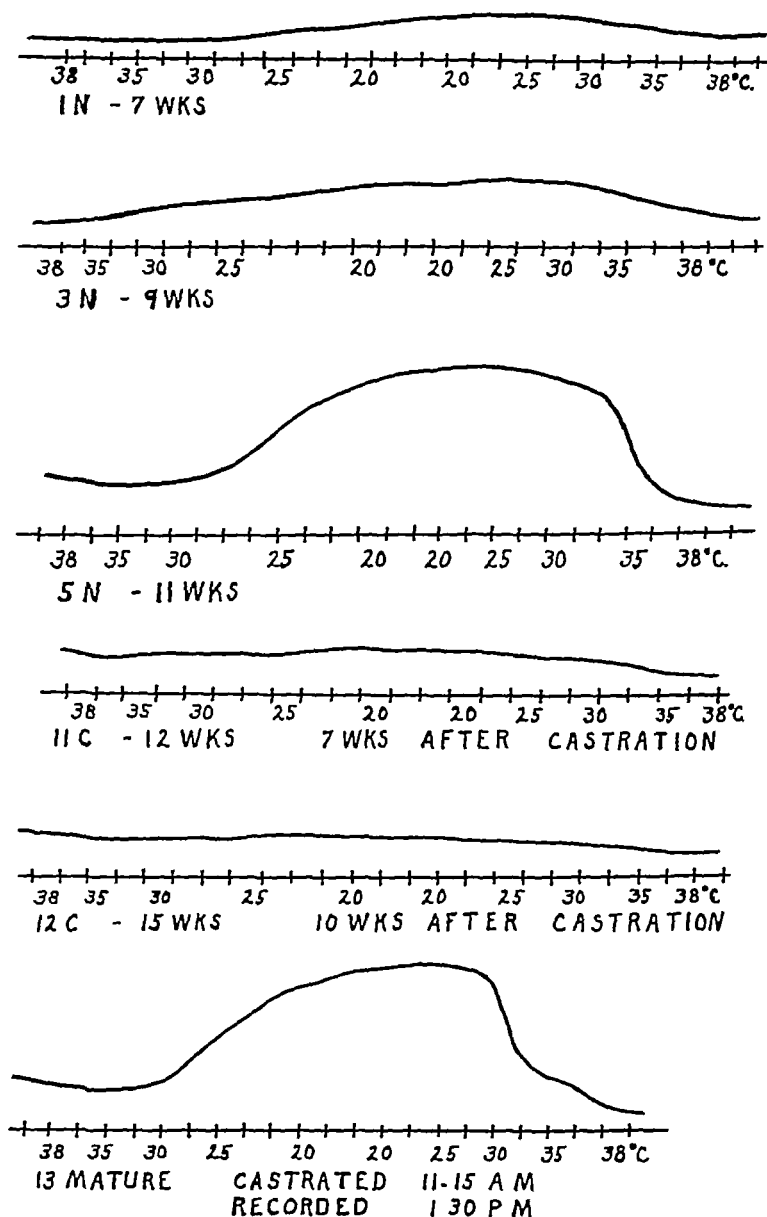


Fig 1

Tracings of kymograph records showing the effect of temperature change on the contractility of the rat scrotum. The time is recorded at two minute intervals on the base line. N = Normal C = Castrate

TABLE I

Development of Testis and Scrotal Sac in Normal Rats and of Scrotal Sac in Rats Castrated at 5 Weeks

Rat No	Age (wks)	Body wt at autopsy g	Testicular wt, g		Stage of spermatogenesis	Combined wt left and right scrotal sacs, g
			Left	Right		
1N	7	112	0.58	0.58	Sperm heads in some tubules	0.24
2N	8	141	0.76	0.72	" " " " "	0.27
3N	9	166	0.90	0.91	Abundant sperm	0.37
4N	10	191	1.10	1.17	Scattered "	0.28
5N	11	220	1.21	1.37	Abundant "	0.43
6N	12	226	1.40	1.43	" "	0.42
7N	14	249	1.27	1.20	" "	0.46
8C	6	96				0.11
9C	8	128				0.07
10C	10	184				0.11
11C	12	220				0.10
12C	15	227				0.18
13C	Mature	230				—

N = Normal. C = Castrate

from 38°C to 20°C at the rate of about 1°C per minute and then raised to 38°C at the same rate. The scrotal movements were thus recorded on a kymograph. The rats were sacrificed immediately upon the completion of the kymograph records. The testes of the normal rats were removed, weighed fresh and fixed in Bouin's fluid, and the scrotal sacs (composed of the peritoneum, internal spermatic fascia, cremasteric skeletal muscle and external spermatic fascia) were cut at the inguinal ring, weighed fresh, and fixed.

*Results* Representative tracings of kymograph records showing the relation of temperature to scrotal contractility are shown in Fig. 1. The rapid testicular development initiated about the 9th week was followed by the ability of the normal rat scrotum to respond readily to temperature change. This ability did not develop in those rats castrated at 5 weeks of age. The presence of the testes in the scrotum is not essential as demonstrated by the fact that the fully developed scrotum of rat 13 responded readily although castration had been performed a few hours earlier. Testicular weights and changes, and scrotal sac weights are presented in Table I. The gradual loss of the thermo-regulatory function of the mature rat scrotum following castration, and the effects of cryptorchidism and male hormone upon scrotal contractility are now being studied in the hope that they may not only be of value in understanding the scrotal mechanism but of assistance in physiological assays of the male hormones.

## Cross-Excitation Between Mammalian Medullated Nerve Fibers After Treatment with Veratrine

T P FLNG AND T H LI

*From the Department of Physiology, Peiping Union Medical College, Peiping*

Dun and Feng<sup>1</sup> have demonstrated with amphibian preparations that contrary to general belief nerve fibers, but more particularly motor nerve endings after treatment with veratrine, give prolonged repetitive discharges to a single stimulus. We have extended their work and found that mammalian motor nerve endings after veratrinization likewise show prolonged repetitive discharges which can be readily recorded from the ventral root. We have furthermore ascertained that in a cat which has received an intravenous injection of a suitable amount of veratrine, such as 2 cc of 0.1% solution, the nerve fibers in the nerve trunk can also show prolonged repetitive firing. In the course of our work we had occasion to make various control tests, some of which dealt with possibilities which at first seemed very remote. One of the tests consisted of stimulating one ventral root bundle and leading off from another ventral root bundle of the same segment and side, both bundles being sectioned centrally at their exit from the cord. In a normal cat, in view of Blair and Erlanger's work,<sup>2</sup> one would naturally expect to lead off nothing but stimulus artifact in this way, but in a veratrinized animal one could not be so sure. In fact we have found that in a veratrinized cat in which the motor nerve endings and the nerve fibers in the sciatic nerve trunk were giving prolonged abundant after-discharges, some discharges could generally be led off from one ventral root when another ventral root was stimulated with a single shock. Such discharges have characteristic long and rather irregular delays in their onset, their shortest latency being about 4 msec and the longest exceeding 30 msec. This long latency, in addition to other reasons, excludes the possibility that the discharges might be due to the escape of the stimulating current from one root to the other, and there is left only one reasonable interpretation that they result from cross-excitation between nerve fibers. The phenomenon appears not so strange, if one recalls that cross-excitation between amphibian medullated nerve fibers in the sciatic nerve trunk after treatment with hypertonic solutions has

<sup>1</sup> Dun, F. T., and Feng, T. P., *Chinese J. Physiol.*, 1940, **15**, 405.

<sup>2</sup> Blair, E. H., and Erlanger, J., *Am. J. Physiol.*, 1932, **101**, 559.

been shown by Kwassow and Naumenko<sup>3</sup> The work of Jasper and Monnier<sup>4</sup> on the transmission of excitation between excised non-myelinated nerves may also be recalled here, and it is interesting to note that the transmission in this case is likewise attended by a long delay For excitability changes in nerve fibers produced by impulses in adjacent nerve fibers see Otani<sup>5</sup> and Katz and Schmitt<sup>6</sup> It is significant that in all cases so far known in which actual cross-excitation between nerve fibers takes place, the nerve fibers have a tendency to enter into rhythmical activity This is true of the preparations used by Kwassow and Naumenko and by Jasper and Monnier, and it is also the case with our veratrinized mammalian nerve An adequate discussion of the subject, however, can only be attempted later in the detailed report

## 11866

### Effect of Foster-Nursing upon Inborn Resistance of Mice to St. Louis Encephalitis

F HOWELL WRIGHT (Introduced by Leslie T Webster)

*From the Laboratories of the Rockefeller Institute for Medical Research, New York*

Previous studies from this laboratory have demonstrated that certain factors which regulate the resistance of mice to infectious disease are inherited<sup>1</sup> Bittner's recent work, however, on the influence of mothers' milk on the susceptibility of mice to cancer indicates that the same sort of foster-feeding test should be applied to the problem of susceptibility to infection<sup>2</sup>

Litters inherently susceptible to St Louis encephalitis were placed with mothers of resistant litters and vice versa within 24 hours of birth As controls, susceptible litters were likewise interchanged among susceptible mothers and resistant litters among resistant mothers respectively Foster-nursing was continued 3 weeks and then each mouse of each litter was inoculated intranasally with 0.03 cc of a 1:10 or 1:100 suspension of mouse-brain St Louis enceph-

<sup>3</sup> Kwassow, D. G., and Naumenko, A. I., *Pflugers' Arch.*, 1936, **237**, 576

<sup>4</sup> Jasper, H. H., and Monnier, A. M., *J. cell comp. Physiol.* 1938, **11**, 259

<sup>5</sup> Otani, T., *Japanese J. Med. Sciences Biophysics*, 1937, **4**, 355

<sup>6</sup> Katz, B., and Schmitt, O. H., *J. Physiol.*, 1940, **97**, 471

<sup>1</sup> Webster, L. T., *J. Exp. Med.*, 1937, **65**, 261

<sup>2</sup> Bittner, J. J., *Am. J. Cancer*, 1940, **30**, 104



litis virus Further controls from litters nursed by their own mothers were likewise inoculated

The results of the tests are shown in Fig 1 Of 104 susceptible young mice nursed by their own mothers, 90% succumbed between the 5th and 7th days and 96.2% by the end of the 3-week period of observation (Fig 1-A) Foster-nursing of 65 susceptibles

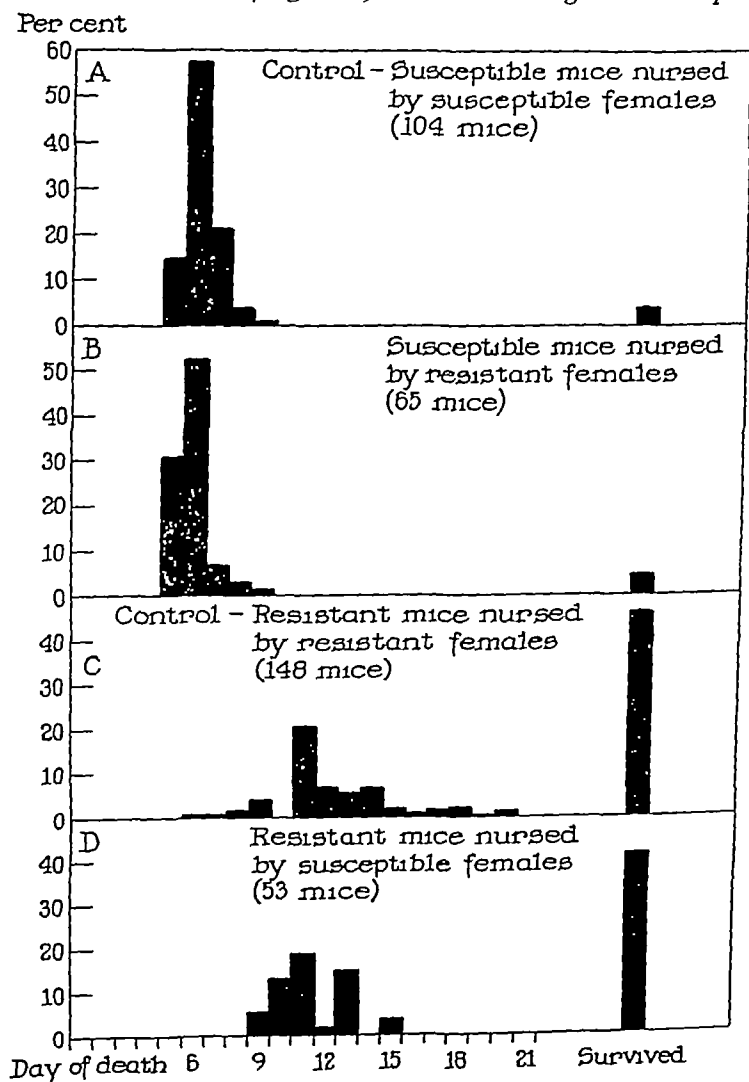


FIG 1

Period of survival of control and foster nursed mice of the virus resistant and virus susceptible strains

by resistant foster mothers gave almost identical results (Fig 1-B) Of 148 resistant young mice nursed by their own mothers, approximately 54% succumbed (Fig 1-C), a figure which is higher than that previously reported and due, in all probability, to the discontinuance of selective breeding for 3 years In contrast to the susceptible mice, the resistants rarely died before the 9th day, at which time nearly all the susceptibles were dead, and most frequently on or about the 11th day Foster-nursing of 53 resistant young by susceptible mothers again produced no change in the amount or distribution of deaths (Fig 1-D)

*Summary* In strains of mice selectively bred for resistance or susceptibility to St Louis encephalitis virus, foster-nursing exerted no effect upon the course of disease or mortality-rate following nasal instillation of virus

11867 P

### Secretion of Inulin by the Kidney of the Crayfish \*

N S RUSTUM MALUF (Introduced by E C Faust )

*From the Department of Zoology, The Johns Hopkins University, Baltimore, and the Department of Tropical Medicine, The Tulane University, New Orleans*

Cumulative evidence indicates that inulin is not secreted by nor passively reabsorbed through the vertebrate nephric tubule (Smith<sup>1</sup>) In vertebrates, the inulin-clearance, therefore, is independent of the concentration of inulin in the plasma and is used as a measure of the rate of glomerular filtration

In an attempt to find whether filtration occurs through the nephron of the crayfish by the use of inulin, the writer obtained most unexpected results as regards the inulin-clearance This is the first invertebrate kidney which has been studied from the standpoint of "clearances" Inulin was measured by a micro-adaptation of the Shaffer-Hartmann-Somogyi method

Nowhere in the crayfish nephron<sup>2</sup> is there a tenuous syncytium such as constitutes the glomerular capsule of the vertebrate nephron

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\* This work was performed largely during the tenure of a Johnston Research Scholarship at The Johns Hopkins University The measurements of inulin clearance were completed in the Department of Tropical Medicine, Tulane University

<sup>1</sup> Smith, H. W., *The Physiology of the Kidney*, New York, Oxford University Press, 1937

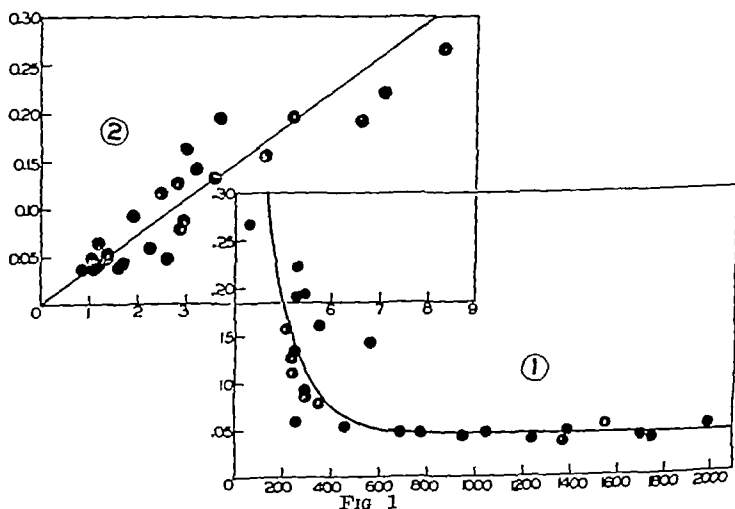
<sup>2</sup> Maluf, N S R, *Zool Jb, Abt f allg Zool u Physiol d Tiere*, 1939, 50, 515

The renal arterioles open freely into the cavernous haemocoel which follows the numerous involutions of the renal epithelium. The kidneys of the crayfish consist of a pair of relatively large nephrons, each of which ends blindly in a highly lobulated coelomosac.

Contrary to the condition in the vertebrate aglomerular kidney, inulin, xylose, and glucose appear in the urine of the crayfish after being injected into the blood stream. This, however, is not proof of filtration because the inulin-clearance is not independent of the concentration of inulin in the plasma. The inulin-clearance plasma-inulin curve (Fig 1), which resembles an adsorption isotherm, shows that inulin is outwardly secreted. This is further indicated by the fact that the shape of the curve in Fig 1 is determined practically entirely by the urine/plasma ratio for inulin (Fig 2).

The sudden decline in the inulin-clearance with augmentation of the plasma-inulin (Fig 1) indicates that inulin is not appreciably filtered. Filtration, therefore, apparently does not occur through the crayfish nephron.

Since the labyrinthal portion of the crayfish nephron is the only tissue of the body which is capable of accumulating and outwardly secreting a number of dyes (cyanol, phenol red, indigo disulfonate, acid and basic fuchsin),<sup>3</sup> it seems probable that inulin, xylose, and



The inulin-clearance (ordinate) in cc per hour as a function of the plasma inulin concentration (abscissa). Each point stands for a single separate crayfish.

The urine/plasma ratio for inulin (ordinate) as a function of the plasma inulin concentration (abscissa). Each point stands for a single separate crayfish, the same individuals as in Fig 1.

glucose are outwardly secreted by the labyrinth. In contrast to the condition in the aglomerular nephron of vertebrates, cyanol, a blue anilin dye, is outwardly and rapidly secreted by the crayfish kidney even when present in low concentrations in the plasma. Evidently the labyrinth has varied secreting capacities.

The maintenance of an internal aqueous and saline steady state, in spite of a constant inward diffusion of water, is known to be due to the unvarying capacity of the crayfish nephron to eliminate urine that is markedly hypotonic to the blood. Since filtration apparently does not occur through this nephron, the hypotonicity cannot be explained by a reabsorption of salts but, like the aglomerular teleost kidney, is evidently due to a secretion of water.

The above facts permit the following conclusions with regard to the physiology of the nephron of the crayfish:

1. Inulin is secreted.
2. Filtration is evidently not an appreciable factor in urine-formation.
3. The manufacture of a hypotonic urine must be due to a secretion of water.

## 11868 P

### Treatment of Cecal and Liver Trichomoniasis in Turkeys by Fever Therapy

MARLOW W. OLSEN AND ENA A. ALLEN (Introduced by R. M. Fraps)

*From the Bureau of Animal Industry, U. S. Department of Agriculture, Beltsville Research Center, Beltsville, Md.*

Since 1937 experimental work has been in progress on the use of fever therapy for the cure of cecal and liver trichomoniasis in turkeys, a widespread disease of domestic fowls caused by the protozoan parasite, *Trichomonas gallinarum*. This organism invades the tissues of the lower digestive tract of birds and in chronic infection produces lesions in the ceca and liver. These lesions closely resemble, and undoubtedly have been often confused with, those produced by *Histomonas meleagridis* in the disease commonly known as black-head. Birds with chronic trichomoniasis are droopy in appearance, refuse to eat, and lose weight. The droppings become quite liquid and light yellow in color. Young birds usually die 4 to 8 days after exhibiting the first symptoms of the disease, although in mature birds the period between the first symptoms and death may be much longer.

Most of the turkeys used in this preliminary study were naturally

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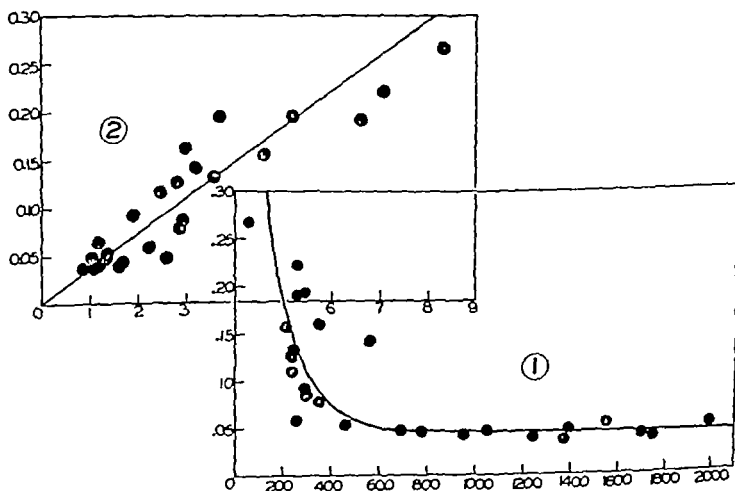


Fig 1

The inulin clearance (ordinate) in cc per hour as a function of the plasma inulin concentration (abscissa). Each point stands for a single separate crayfish.

Fig 2

The urine/plasma ratio for inulin (ordinate) as a function of the plasma inulin concentration (abscissa). Each point stands for a single separate crayfish, the same individuals as in Fig 1.

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infected and ranged from 3 to 18 months in age. At the beginning of the work the disease was diagnosed by the external symptoms noted above, and by microscopic examination of the cecal discharges.

Recently it has been found that by making an incision into the abdominal cavity, and with the aid of a strong light, it is possible to observe the liver and ceca directly for lesions. The direct observation of lesions and identification of trichomonads in cecal droppings have made certain the elimination from treatment of birds exhibiting external symptoms similar to those of trichomoniasis but actually due to other causes.

Birds undergoing treatment were placed in a thermostatically controlled cabinet for periods ranging from 1 to 2 hours. The internal body temperature of the bird was raised from 2 to 6 degrees above the normal of 106.5°F by maintaining an air temperature within the cabinet of approximately 104°F, and a relative humidity of 60 to 70%. The duration of treatment depended upon the physical condition of each bird and its ability to withstand the increased temperature. Usually three treatments at intervals of every other day were sufficient to check the disease, although as many as 6 treatments were necessary in a few more advanced cases. After treatment the birds were placed in wire-bottomed cages at room temperature.

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Tulane University	January 26, 1940
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WESTERN NEW YORK	
University of Rochester	February 17, 1940

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11221 P

Relief of Hypochromic Anemia in Dogs with Synthetic Vitamin  
B<sub>6</sub> Influence of "Filtrate Factors"\*

HARRY J BORSON AND STACY R METTIER (With the technical  
assistance of Miss Alice McBride )

*From the Divisions of Medicine and Pathology, University of California Medical  
School, San Francisco*

Fouts, Helmer, Lepkovsky and Jukes<sup>1</sup> maintained dogs on a synthetic diet supplemented with all the known members of the vitamin B complex except B<sub>6</sub> (Factor 1, rat, anti-dermatitis vitamin), and

---

\* Aided by a grant from the Christine Breen Fund for Medical Research

<sup>1</sup> Fouts, P J, Helmer, O M, Lepkovsky, S, and Jukes, T H, *J Nutrition*, 1938, 10, 197

produced severe hypochromic microcytic anemia which was rapidly relieved with a rice-bran eluate rich in B<sub>6</sub>. They later<sup>2</sup> reproduced the result with crystalline B<sub>6</sub> isolated from natural sources.<sup>3</sup> In the present report it is shown that synthetic B<sub>6</sub> (2-methyl, 3-hydroxy, 4,5-di-[hydroxy-methyl] pyridine)† will replace the natural material in the relief of this anemia.

Adult mongrel male dogs varying in weight from 7.5 to 21 kg were maintained on a purified diet of the following composition: casein (extracted for a week with acidulated water) 37%, sucrose 34.8%, cotton-seed oil 12%, lard 9%, cod-liver oil 3%, bone ash 2.4%, and salt mixture (Cowgill) 1.8%. The original salt mixture was modified by doubling the amount of ferric citrate. Supplements of various members of the vitamin B complex were given (per kg per day) as follows: thiamin chloride 50 µg, riboflavin‡ 50 µg, nicotinic acid‡ 1 mg, liver extract§ 0.06 ml. The liver extract was prepared from a crude liver concentrate by repeated treatment with fuller's earth to remove the adsorbable components. It was shown by rat assay to be rich in Factor 2 (rat growth factor) but practically free of B<sub>6</sub>. Diet and water were given *ad lib*.

Four dogs, Nos. 74, 164, 165, and 188, maintained on this regime for from 120 to 135 days, developed hypochromic microcytic anemia with red blood cell counts of 5.78, 5.10, 4.95, and 4.91 million, hemoglobins of 6.3, 7.5, 7.2, and 5.9 g, mean corpuscular volumes of 51, 47, 49.5 and 45.8 cubic micra respectively. When the diet was started the red counts were 6.71, 6.87, 6.43, and 6.44 million, hemoglobins were 15.6, 14.7, 15.3, and 14.4 g, M.C.V. were 64, 72, 75, and 64 cubic micra. Dog 74 was then given natural B<sub>6</sub> (obtained from Dr. Samuel Lepkovsky) in doses of 60 µg per kg per day. A slight reticulocytosis occurred (4% on the fifth day) and on the thirtieth day the red count was 7.0 million and the hemoglobin 11.6 g. Dog 164 was given synthetic B<sub>6</sub> in doses of 60 µg per kg per day. The reticulocytes increased on the second day and reached a peak of 6.8% in 6 days. In 14 days the red count had risen to 5.5 million, the hemoglobin to 10.2 g, and the M.C.V. to 61.8 cubic micra. On the thirty-fifth day the red count was 6.19 million, hemoglobin 12.6 g and M.C.V. 66.3 cubic micra. Dog 165 was given 60 µg of synthetic

† Supplied by Dr. D. F. Robertson, Merck and Co., Rahway, N.J.

2 Fouts, P. J., Helmer, O. M., and Lepkovsky, S., *Proc. Soc. Exp. Biol. and Med.*, 1939, **40**, 4.

3 Lepkovsky, S., *J. Biol. Chem.*, 1938, **124**, 125.

‡ Supplied by the Galen Co., Berkeley, Calif.

§ Made and assayed by the Galen Co., Berkeley, Calif., and the Vitab Corp., Emeryville, Calif.

B<sub>6</sub> per kg per day After a slight increase in reticulocytes to 4.6%, the red count and hemoglobin increased by the fourteenth day to 5.78 million and 9.5 g respectively. The M C V was 60.5 cubic micra. On the thirty-fifth day the red count was 6.24 million, hemoglobin 11.4 g and M C V 61.3 cubic micra. Dog 188 was given 60 µg synthetic B<sub>6</sub> per kg per day and in 14 days the red count was 6.1 million and the hemoglobin 9.5 g.

A control animal was maintained on the same regime with the addition of vitamin B<sub>6</sub>, first in the form of a rice-bran eluate§ (free of the "filtrate factors") and later as synthetic B<sub>6</sub> in the amount of 60 µg per kg per day. He did not develop hypochromic anemia, but after several months he developed very mild normochromic normocytic anemia. This was found to be the result of a mild deficiency of the liver extract supplying the non-adsorbable "filtrate factors." Two animals maintained on the diet without addition of the filtrate but with 60 µg per kg per day of B<sub>6</sub> developed moderate anemia of the normochromic normocytic type.

At the start of the experiment the requirement of the dog for the non-adsorbable factors other than nicotinic acid was not known and we later found that the amounts given were insufficient. This prevented the complete relief of the hypochromic anemia by the administration of B<sub>6</sub>, as the following data show. Dog 74, as noted above, responded rapidly to crystalline B<sub>6</sub>. After the thirty-first day of treatment, however, the hemoglobin failed to rise further and during the next 14 weeks it remained at about the same level, fluctuating between 10.7 and 11.6 g. The red count slowly dropped from 7.0 to 5.4 million. Substitution of synthetic for natural B<sub>6</sub> in both the same and double dosage had no effect. Addition of rice-bran eluate (containing only adsorbable factors) and increase of riboflavin had no effect. When finally the liver filtrate level was tripled, the hemoglobin rose within 28 days to 13.9 g, the red count to 6.28 million and the M C V from 51.5 to 73. An adequate supply of a factor (or factors) present in the filtrate after exhaustive adsorption with fuller's earth is apparently necessary for complete relief of the anemia resulting from deficiency of B<sub>6</sub>. This interrelationship is being further investigated. The filtrate used is known to be a rich source of the chick anti-dermatitis factor and the rat growth vitamin, but it cannot be said that the factor required by the dog is identical with either of these.

In addition to the anemia, the dogs deficient in B<sub>6</sub> presented other manifestations. All except one lost weight and all exhibited marked listlessness and asthenia. The lassitude was not entirely the result of the anemia since it improved remarkably after only a few days



of treatment with B<sub>6</sub> before the hemoglobin had appreciably increased

*Conclusions* Synthetic vitamin B<sub>6</sub> relieves the hypochromic microcytic anemia produced in dogs deficient in this factor. An adequate supply of the non-adsorbable fraction of the vitamin B complex is necessary for the complete disappearance of this anemia.

## 11222 P

### Response of Hypophysectomized Rats to Highly Purified Extracts of Pregnant Mare Serum

R. I. PENCHARZ, H. H. COLE AND H. GOSS

From the Endocrine Laboratory, Department of Obstetrics and Gynecology, University of California Medical School, San Francisco, and the Division of Animal Husbandry, University of California, Davis

Goss and Cole<sup>1</sup> have shown that extracts of mare serum can be prepared testing 4000 to 7000 R. U. per mg total solids (40,000 to 70,000 R. U. per mg nitrogen). The question arises regarding the nature of the hormone present in these extracts as compared to that in untreated mare serum. Evans, *et al.*,<sup>2</sup> and Hellbaum<sup>3</sup> have reported that more than one gonadotropic hormone is present in mare serum though these reports have not been confirmed. In the first mentioned paper the male rats showing only a Leydig tissue response were possibly sacrificed too soon to expect an effect upon the seminiferous epithelium. Further, had the time of autopsy of the females been delayed beyond 72 hours corpora lutea might have been encountered following the treatment with so-called FSH fractions. The extracts prepared by these authors were relatively crude preparations. We were interested, therefore, in determining whether or not highly purified materials would give similar evidence of fractionation of gonadotropic hormones. A fraction giving pure follicular stimulation in the female with little or no effect on the interstitial tissue of the male would give evidence of a purified follicle stimulating hormone whereas a converse relationship would indicate the presence of a luteinizing hormone.

*Experimental* Two extracts were used one, No 15-19-2 tested

<sup>1</sup> Goss, H., and Cole, H. H., *Endocrinology*, 1940, **20**, 244

<sup>2</sup> Evans, H. M., Korpi, K., Simpson, M. E., and Pencharz, R. I., *Univ. of California Publication in Anatomy*, 1936, **1**, 275

<sup>3</sup> Hellbaum, A. A., *Am. J. Physiol.*, 1937, **119**, 331

TABLE I.  
Results on Males Injected with 20 R U of No 15 19 2 Daily for 20 Days

Rat	Hypophysectomized		Treatment		Autopsy			
	Age in days	B W (g)	Age in days	Daily dose, rat units	Age in days	Seminal		
						Testes (mg)	vesicles (mg)	Prostate (mg)
G6953 C	40	112	75	20	96	660	440	418
W0952 C	40	118	75	20	96	681	433	412
BH6941 C	40	114	75	20	96	597	397	351
B6950 C	40	112	None, hyp'd control		96	240	9	25
B 6926 C	Normal unoperated	123	Uninjected 0		40	1250	20	90

35,000 and the other, No 17-76-4, 40,000 R U per mg nitrogen \* Four to 6 female rats, hypophysectomized on the 23rd day of age, were used at a given level for each extract. The intraperitoneal administration of a total of 30 R U over a 3-day period beginning on the 30th day of age produced ovaries averaging 116 mg for No 15-19-2 and 127 mg for No 17-76-4, on the 5th day after the initial injection. Sixty rat units produced ovaries weighing 122 mg and 101 mg, respectively. Estrous smears were encountered in all test animals. The macroscopic observation of many ripe follicles with some corpora lutea was confirmed microscopically.

The extracts were also tested in hypophysectomized male rats. The results on one extract, No 15-19-2, are shown in Table I. There was a strong interstitial cell response as is indicated by the response of the accessory organs. Two rats treated similarly with No 17-76-4 gave like results.

Thus it is seen that these extracts containing much less inert material than any previously reported for mare serum give a good follicular response in the hypophysectomized female and a strong interstitial cell response in the hypophysectomized male. In other words, in spite of the high degree of purification attained in the present extracts, no evidence was secured to support the view that mare serum contains 2 distinct hormones, one specifically affecting the interstitial tissue of the ovary and the Leydig tissue of the testis, and a second causing follicular growth in the female and germinal tissue development in the male.

\* The potency of the extracts was determined in normal immature rats according to the method of Cole, Guilbert and Goss.<sup>4</sup>

<sup>4</sup> Cole, H. H., Guilbert, H. R., and Goss, H., *Am. J. Physiol.*, 1932, 102, 227.

## 432 HIGHLY PURIFIED EXTRACTS PREGNANT MARE SERUM

of treatment with B<sub>6</sub> before the hemoglobin had appreciably increased

*Conclusions* Synthetic vitamin B<sub>6</sub> relieves the hypochromic microcytic anemia produced in dogs deficient in this factor. An adequate supply of the non-adsorbable fraction of the vitamin B complex is necessary for the complete disappearance of this anemia

11222 P

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*Experimental* Two extracts were used one, No 15-19-2 tested

<sup>1</sup> Goss, H, and Cole, H. H, *Endocrinology*, 1940, **26**, 244

<sup>2</sup> Evans, H. M., Korpi, K., Simpson, M. E., and Pencharz, R. I., *Univ. of California Publication in Anatomy*, 1936, **1**, 275

<sup>3</sup> Hellbaum, A. A., *Am J Physiol*, 1937, **119**, 331

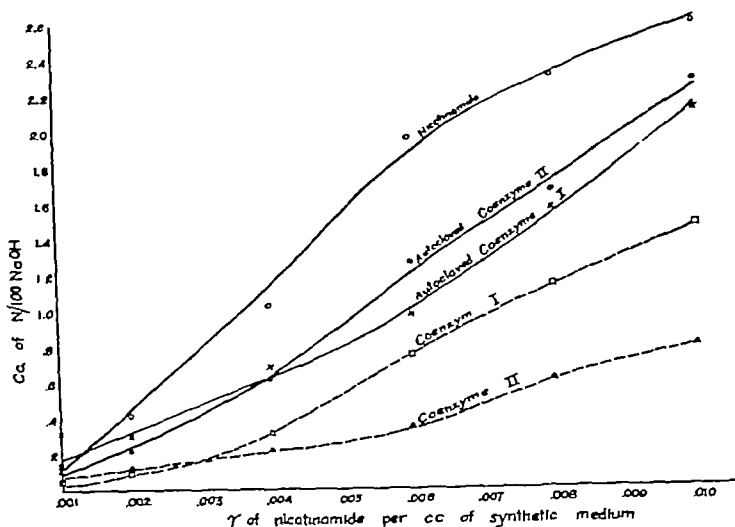


FIG 1

coenzyme I, coenzyme II, and autoclaved coenzymes I and II. It will be noted that nicotinamide is the most active growth-promoting substance, and coenzyme I is somewhat more active than coenzyme II. After the coenzymes are autoclaved (20 pounds, 30 minutes) the activity of each is markedly increased. Under certain conditions of heating it is possible to bring the activity up to that of an equivalent amount of nicotinamide. The results discussed above are obtained when the culture is titrated at the end of 4 days' incubation. If a shorter period is used the results are more striking since the coenzymes exhibit a lag period similar to that reported by us for the acid.

These findings are incompatible with the theory that nicotinamide functions solely as a building block for coenzyme I or coenzyme II. If nicotinamide is more active than an equivalent amount of either of the pyridine-containing coenzymes, it must have some importance other than, or in addition to, being a component of the known coenzymes. Metabolic experiments have confirmed this hypothesis although the details of this new mechanism are not yet known.<sup>4</sup>

Because of the rapid accumulation of evidence for the great importance of nicotinamide and related compounds in the nutrition and metabolism of various living forms there have been numerous attempts to develop methods for the determination of these compounds in biological materials.

<sup>4</sup> Dorfman, A., Koser, S. A., and Saunders, F., *Science*, 1939, 90, 544.

# Quantitative Response of the Dysentery Bacillus to Nicotinamide and Related Compounds \*

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In a previous report we showed that many strains of dysentery bacilli require nicotinamide or certain closely related compounds for growth <sup>1</sup> We have also studied the growth-promoting activity of a number of compounds structurally related to nicotinamide <sup>2, 3</sup> It is the purpose of this paper to report certain quantitative studies on the activity of nicotinic acid, nicotinamide, coenzyme I and coenzyme II These studies were carried out in an attempt to learn the mechanism of action of these compounds in promoting growth of dysentery bacilli The results were also used for the development of a method for the estimation of nicotinamide and related compounds in blood

It has been generally assumed that nicotinic acid is converted to nicotinamide and that the latter is converted to one or both of the pyridine-containing coenzymes We have presented evidence for the conversion of the acid to the amide, based on the relative activity of the two substances in promoting growth of dysentery bacilli <sup>3</sup> It was found that when the acid is used as a growth-promoting substance there is a very definite lag in growth as compared with that obtained when the amide is used

If nicotinamide is converted to one of the known pyridine-nucleotides, one or both of them must have a growth-promoting activity greater than or equal to that of an equivalent amount of nicotinamide

In order to determine the relative activities of the various substances the titration method previously described by us was employed <sup>†</sup> Figure 1 indicates the relative activity of nicotinamide,

\* This work was aided by a grant from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago and a grant from the Committee on Scientific Research of the American Medical Association

<sup>1</sup> Koser, S. A., Dorfman, A., and Saunders, F., *Proc. Soc. Exp. Biol. and Med.*, 1937, **38**, 311

<sup>2</sup> Dorfman, A., Koser, S. A., and Saunders, F., *J. Am. Chem. Soc.* 1938, **60**, 2004.

<sup>3</sup> Dorfman, A., Koser, S. A., Reames, H. R., Swingle, K. F., and Saunders, F., *J. Inf. Dis.*, 1939, **65**, 163

<sup>†</sup> We are indebted to Professor Otto Warburg for a sample of pure coenzyme II and to Dr. A. Axelrod for a sample of pure coenzyme I prepared in Euler's laboratory

TABLE I.  
Effect of Autoclaving on Amount of Nicotinamide Found in a Sample of Human Blood.

Determination No	Dilution of filtrate, cc	N amide added per cc, $\gamma$	Method of sterilization	Value found, $\gamma$ per cc
1	020	—	Filtered	6.1
	030			6.2
2	025	5	"	10.3
	015			11.2
3	025	—	Autoclaved	7.5
	015			8.4
4	015	5	"	13.2
	010			12.0

Table I illustrates the results obtained on a normal sample of blood. The value obtained for total activity expressed as nicotinamide is dependent upon the method of sterilization employed. In this experiment the same blood was employed for all analyses. It will be noted that the activity is markedly increased by heat treatment. More drastic treatment resulted in little increase in activity. This increase in activity is to be expected on the basis of the results reported in the first part of this paper, since it is known that most of the nicotinamide present in blood exists in the combined form.

Table I also illustrates the type of results that can be obtained by running the samples at different dilutions, and the recovery of nicotinamide when added to the whole blood. We have occasionally experienced difficulties in obtaining checks at different dilutions. The explanation for these difficulties was not found.

Kohn<sup>8</sup> has pointed out that the amount of "V" substance present in human blood is a function of the hematocrit. In an earlier report we showed that all of the activity for the dysentery bacilli resides in the erythrocytes.<sup>9</sup> Table II shows the results obtained with 5 different bloods both before and after autoclaving, together with hematocrit values. The number of samples analyzed by us is insufficient to permit drawing any definite conclusions concerning the relation between hematocrit and nicotinamide.

The results given in Tables I and II are typical for normal human samples.

**Summary** By means of a titration method for estimation of bacterial growth it was found that growth is proportional to the quantity of nicotinamide present. Nicotinamide is more active than an

<sup>8</sup> Kohn, H. I., and Bernheim, F., *J. Clin. Invest.*, 1939, 18, 585.

<sup>9</sup> Dorfman, A., Horwath, M. K., Koser, S. A., and Saunders, F., *J. Biol. Chem.*, 1939, 128, xx.

The method which we have used is essentially the same as that described by us in a previous publication<sup>5</sup>. It consists in determining the amount of acid produced by a dysentery culture to which a definite dilution of a blood filtrate is added. The nicotinamide equivalent is determined by comparison with a standard curve (Fig. 1). The dilutions of the blood sample were made so that a minimum of 0.2 cc and a maximum of 0.4 cc was added to each tube containing 4.5 cc of basal medium. The final volume was made up to 5.0 cc. The tubes were incubated for 4 days at 37° and were then titrated with 0.01 *N* NaOH to a standard color of brom thymol blue. All blood samples were run at 2 different dilutions. Blank determinations were made on both inoculated and uninoculated controls. A series of nicotinamide standards was run with each set of blood samples. All determinations were run in triplicate.

The addition of a mixture of all of the available other known growth substances resulted in no increase in acidity, thus indicating that this test is specific for nicotinamide and related substances.<sup>6</sup> Schmelkes<sup>5</sup> has reported that thiazole-5-carboxylic acid is able to substitute for nicotinamide in promoting growth of dysentery bacilli. We have found that the activity of this compound is approximately one-hundred-thousandth that of nicotinamide, and therefore even if it did occur naturally it would not interfere with this assay.<sup>6</sup>

We first attempted to prepare our samples for assay by means of an acetone extract. It was found, however, that blood samples prepared in this manner gave results which were lower than those obtained with a water extract. Euler<sup>7</sup> has used an acetone method to determine the amount of free nicotinamide in blood. He obtained values of about 1  $\gamma$  per cc when he used the cyanogen bromide method for determination of the nicotinamide. Our values when we used acetone for the preparation of the samples were somewhat higher, the normals averaging about 3  $\gamma$  per cc. This difference is probably due to the fact that we employed 3 volumes of acetone, while Euler used 10 volumes. The latter quantity would extract less of the coenzymes.

The method used in most of our studies consisted in adding one volume of whole blood to 3 volumes of water within 10 minutes after the samples were drawn. This mixture was then heated to 70° for about 10 minutes and the coagulum filtered off. The preliminary heating is necessary to destroy the enzymes in blood which hydrolyze the coenzymes. The supernatant liquid is then ready for assay.

<sup>5</sup> Schmelkes, F. C., *Science*, 1939, **90**, 113.

<sup>6</sup> Koser, S. A., Dorfman, A., and Saunders, F., *Proc. Soc. Exp. Biol. and Med.* 1940, **43**, 391.

<sup>7</sup> Euler, H. V., and Schlenk, F., *Klin. Woch.*, 1939, **18**, 1109.

Radio-chloride has a half life of approximately 35 minutes and lithium a half life of 1/25 second. Thus lithium chloride was employed as a target for bombardment by the beam in the cyclotron of the Departments of Chemistry and Physics. Because of the brief radioactivity of the lithium the salt afforded a soluble chloride in which only the latter ion was radioactive. The lithium chloride was dissolved in sterile water and a drop or two of 1/10 N NaOH added to insure slight alkalinity. The solution was boiled gently for sterilization and then injected intravenously at the desired moment. The injections were made within 45 minutes after the material was received from the cyclotron and the observations completed within 3 hours. Radioactivity was determined by holding approximately 1 to 3 cc of the fluid to be tested in cellophane sacs 1 to 1.5 cm from the aluminum foil-covered aperture of a quartz-fiber Lauritsen electro-scope. Proper control observations were of course made upon the "background" of the room in which the readings were made and upon blood, urine and gastric juice samples before injection of the radio-chloride.

**Experiment I** Female dog An. Wt. 10 kg with large cannulated gastric pouch. After several feedings of cooked lean meat vigorous pouch secretion developed and was collected every 10 minutes in Soxhlet flasks suspended below the cannula. These samples varied from 1 to 1.75 cc per 10-minute period with free acid varying between 100 and 150 clinical units and combined acid 20 to 30 clinical units. At the termination of one period 500 mg of radioactive lithium chloride dissolved in 10 cc of water were injected intravenously in a hind extremity. The volume of juice secreted during the next 10-minute period equalled 3 cc and when tested was found to be strongly radioactive. Similar samples collected at 10-minute intervals for the next 130 minutes each showed radioactivity.

**Experiment II** Male Dog Gyp. Wt. 10 kg with large cannulated gastric pouch. Procedure as in Experiment I. In this case 3 cc were secreted during the 120 seconds following the termination of the injection of lithium chloride. This sample was tested separately and found to be strongly radioactive. Tests were also made on blood and urine samples, the results are indicated in the accompanying graph.

**Experiment III** Patient, Mrs. H., age 58, Wt. 50 kg, thin and emaciated, 8 days postoperative cholecystectomy for cholecystitis and cholelithiasis. A Levine tube was passed into the stomach after 18 hours' starvation and gastric lavage performed with water, all of the latter being withdrawn. One milligram of histamine was injected subcutaneously and 10 minutes later gastric aspirations were begun,



TABLE II.  
 Nicotinamide Content of Human Blood

Sample	Dilution of filtrate	Method of sterilization	Value found, $\gamma$ /cc whole blood	Hematocrit
A	03	Filtered	60	42 0
	02		54	
A	03	Autoclaved	80	42 0
	02		70	
B	03	Filtered	58	46.5
	02		55	
B	03	Autoclaved	.88	46 5
	02		92	
C	03	Filtered	64	53 0
	02		72	
C	03	Autoclaved	91	53 0
	02		.97	
D	03	Filtered	45	41 0
	02		52	
D	03	Autoclaved	83	41.0
	02		85	
E	03	Filtered	58	46 0
E	03	Autoclaved	75	46 0

equivalent amount of either pyridine-containing coenzyme Hydrolysis increases the activity of the latter, indicating that the function of nicotinamide is not based entirely on synthesis to either of the known coenzymes A method has been developed for determining nicotinamide and related substances in blood The values obtained are higher if autoclaved blood is used.

## 11224

### Rapidity of Passage of Chloride Ion from Blood into Gastric Juice of Stimulated Stomach \*

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In order to study the passage of chloride from the blood into the acid gastric juice of the stimulated stomach, chloride ions were "tagged" by rendering them radioactive. Thus radioactivity detected in the juice would signify that such ions if previously injected into the blood had been brought through the gastric mucosa

\* These observations are incidental to a study of achlorhydria in gastric carcinoma supported by grants from the National Advisory Council on Cancer, Washington, D C, and The International Cancer Research Foundation, Philadelphia, Pennsylvania

† Research Assistant, International Cancer Research Foundation Grant

As a control for the method, radioactive phosphorus in the form of sodium phosphate was injected intravenously into three pouched dogs. The blood was observed to be radioactive for over 45 minutes but the gastric pouch juices secreted during this period exhibited no radioactivity, according to the criteria adopted in these experiments.

*Summary* The foregoing observations show that "tagged" chloride ions when injected into the general circulation are identified in the acid gastric juice of the stimulated stomach in dog and in man as quickly as 60 to 120 seconds after injection. Presumably at least some of these ions in the juice were derived from the HCl present. Tagged chloride ions continued to circulate in the blood for more than one hour after intravenous injection and were transported into the gastric juice continuously during this period. Such ions did appear in the urine but were detected at irregular intervals and in relatively low concentration.

## 11225

### Availability of Staphylococcal Antitoxin After Intramuscular Injection into Normal Monkeys and Men \*

CHARLES WEISS (With the assistance of Lucy D. Bergman)

*From the Research Laboratories of the Mount Zion Hospital, San Francisco, Calif.*

The increasing use of staphylococcal antitoxin<sup>1, 2</sup> makes it advisable to study its rate of absorption and concentration in the blood after injection of therapeutic doses. In accordance with previous work on diphtheric antitoxin<sup>3</sup> and antipneumococcal serums,<sup>4</sup> daily estimations were made of the titers of 6 "normal" human subjects who were not suffering from staphylococcal infections and of 9 normal monkeys (*M. mulatta*) after intramuscular injections of Squibb's concentrated antitoxin. Some of each group were injected into the gluteal muscle with 1000 units per kg, while others received 2 such doses, 24 hours apart (Table I). *Alpha* antistaphylocolytic serum

\* Aided by a grant from the Committee on Therapeutic Research, Council on Pharmacy and Chemistry, of the American Medical Association.

<sup>1</sup> Baker, L. D., and Shands, A. R., *J. A. M. A.*, 1939, **113**, 2119.

<sup>2</sup> Stookey, P. F., and Searpellino, L. A., *So. Med. J.*, 1939, **32**, 173.

<sup>3</sup> Glenny, A. T., and Hopkins, B. F., *J. Hygiene*, 1924, **22**, 12, 36, 208, 222.

<sup>4</sup> Finland, M., and Brown, T. W., *J. Immunol.*, 1938, **35**, 245.

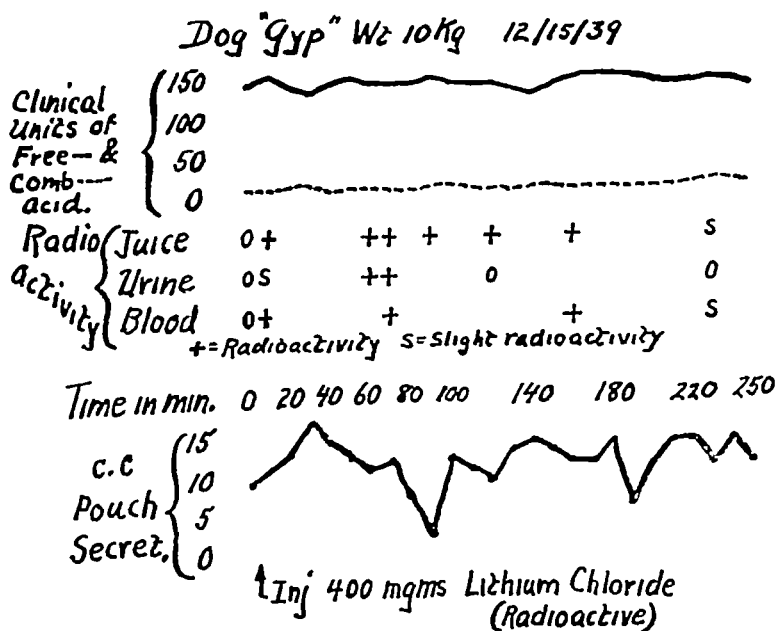


FIG 1

Graph of Exp II described in text showing appearance time of radioactive chlorine in pouch gastric juice subsequent to its injection into an extremity vein. Persistence of the "tagged" ions in the blood and their presence in the urine are also indicated.

and continued at similar intervals for 90 minutes. Following the third aspiration which yielded a juice containing 25 clinical units of free acid and combined acid of 12 clinical units, 400 mg of radioactive lithium chloride dissolved in 10 cc of water were injected intravenously in the arm. During the 120 seconds following the termination of this injection  $1\frac{1}{2}$  cc of gastric juice were aspirated which showed radioactivity. Six subsequent samples aspirated at 10-minute intervals all showed radioactivity. Five cc of blood withdrawn from the antecubital vein 1 minute and 70 minutes respectively following injection showed radioactivity. Prior to the experiment the patient voided. At the termination of the experiment the patient voided 36 cc. Three cc of this urine showed radioactivity but approximately one-half as intense as the last sample of 3 cc of gastric juice tested.

Experiment IV. Patient, St, white male, 23, one week after bilateral herniorrhaphy. Procedure as in Exp III. 3 cc of gastric juice aspirated 60 to 90 seconds following the termination of the injection of radio chloride showed radioactivity. The free acid in this sample equaled 110 clinical units, the combined acid, 17 clinical units.

As a control for the method, radioactive phosphorus in the form of sodium phosphate was injected intravenously into three pouched dogs. The blood was observed to be radioactive for over 45 minutes but the gastric pouch juices secreted during this period exhibited no radioactivity, according to the criteria adopted in these experiments.

**Summary** The foregoing observations show that "tagged" chloride ions when injected into the general circulation are identified in the acid gastric juice of the stimulated stomach in dog and in man as quickly as 60 to 120 seconds after injection. Presumably at least some of these ions in the juice were derived from the HCl present. Tagged chloride ions continued to circulate in the blood for more than one hour after intravenous injection and were transported into the gastric juice continuously during this period. Such ions did appear in the urine but were detected at irregular intervals and in relatively low concentration.

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<sup>3</sup> Glenn, A. T., and Hopkins, B. E., *J. Hygiene*, 1924, **22**, 12-36, 208-222.

<sup>4</sup> Finland, M., and Brown, I. W., *J. Immunol.*, 1938, **35**, 245.

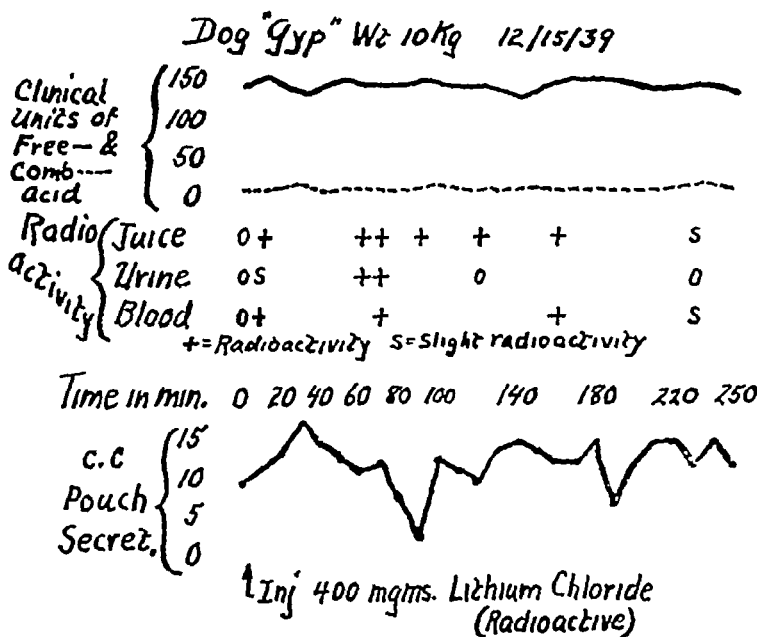


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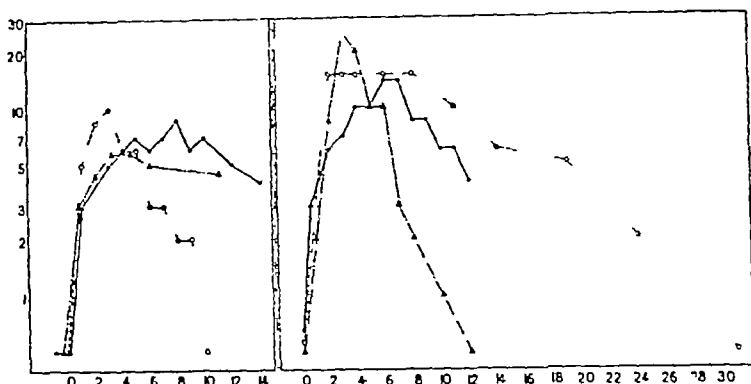


FIG 1.

Rate of absorption of antistaphylococcal antitoxin after intramuscular injection into "normal" human subjects. Left graphs, 3 persons injected with 1000 units per kg, right graphs, 3 injected with 2000 units per kg. Ordinates logarithms of units of *alpha* antistaphylolysin per cc of serum, abscissae, days after injection.

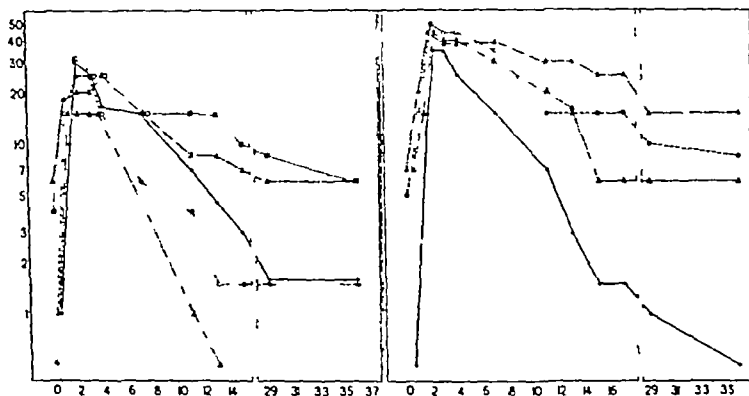


FIG 2.

Rate of absorption of antistaphylococcal antitoxin after intramuscular injection of normal monkeys (*M. mulatta*). Left graphs, 5 monkeys injected with 1000 units per kg. Right graphs, 4 monkeys injected with 2000 units per kg. Ordinates as in Fig 1.

titers were determined by the method of the National Institute of Health<sup>5</sup>

Figs 1 and 2 show striking uniformity in the rates of absorption of *alpha*-antistaphylolysin. Semi-logarithmic curves present 3 phases. In humans, phase A, the stage of absorption, lasted 3 to 8 days. Phase B, the period of maintenance of maximal serum titers, 1 to 6 days, the average being 3 days. Phase C, the stage of elimination of antibody,

<sup>5</sup> National Inst. Health, Washington, D C, B 1199, Jan., 1936, U S Standard Staphylococcus Antitoxin.

TABLE I  
Summary of Data on Normal Monkeys and Persons Injected Intramuscularly with Varying Doses of Concentrated Staphylococcal Antitoxin (Squibb) and Rabbits\* Injected Intravenously with Diphtheric Antitoxin

Animal or patient No	Wt, kg	Plasma volume, cc	Units of antitoxin per kg	Max. increments of serum titers (Units alpha antistaphylo lysin per cc)	Sums of conc of antibody 9 days (Arens)	Avg of areas	Antibody available in total plasma vol.	Avg
Normal monkeys ( <i>M. mulatta</i> )								
229	3.0	182.0	1000	25	110	113.5	17,800	19,160
253	3.4	183.6	1000	26	131		24,050	
243	2.5	135.0	1000	14	86		11,600	
244	2.75	148.5	1000	29	137		20,340	
252	4.0	216.0	1000	19	103		22,000	
254	3.7	145.8	2000	35	170	233.8	24,800	32,320
230	2.6	140.0	2000	45	278		39,030	
255	2.0	103.0	2000	38	270		29,160	
251	3.2	172.8	2000	38	217		37,500	
"Normal" human subjects								
1 MR (g)	57	3,073	1000	8.5	48	43	147,700	141,550
2 RG (n)	66	3,564	900	6	38		135,400	
3 NC (c)	56	3,024	2100	15	101	93	305,420	288,115
4 JF (m)	59	3,188	2000	25	85		270,810	
83 f	3.24	129.6	231.0	5.25	13.03		1,558	
109 f	2.84	113.6	264.0	7	15.90		1,806	
110 f	2.81	112.4	267.0	8	15.07		1,694	
94 y	0.91	86.4	824.0	29	34.17		1,244	
84 y	0.40	16.0	187.0	16	79.24		1,268	

(g) gastric ulcer, male, age 59

(n) arthritis, male, age 31.

(c) non specific conjunctivitis, male, age 25

(m) moron, male, age 17

\*Illustrative data selected from tables of Glenn and Hopkins

y very young animals.

f full grown, almost mature rabbits

therefore, irrespective of the maximal titers or the duration of elimination, the sum of the concentrations of antibody during a given period of time (area under the curve) or the antibody content of the total plasma volume was directly proportional to the dose injected per kg weight

This new procedure was also applied to data selected from Glenny and Hopkins<sup>3</sup>. They injected a constant dose of diphtheric antitoxin (750 units) into each of 5 rabbits weighing from 0.400 to 3.240 kg. Although the maximal titers and duration of elimination varied, as reflected in the sums of the concentrations of antibody during 9 days (12 to 79), nevertheless the antibody available in the total plasma was practically identical in all animals. In other words, with a given total dose injected, the turnover of antibody in the plasma is the same.

Preliminary observations on *beta* antistaphylolysin<sup>4</sup> revealed a uniform rate of absorption but the serum titers reached were very small, never exceeding 1 to 6 units, in normal persons and monkeys respectively. This may be explained by the low concentrations in commercial antitoxin which did not exceed 15 and 80 units per cc in Lederle's and Squibb's antitoxin respectively.

*Summary and Conclusions* After intramuscular injection of staphylococcal antitoxin the rate of absorption is quite uniform in normal monkeys and humans. The antibody available in the total plasma-volume during a given period of days after injection of a specified dose (per kg weight) may be determined from the area under the curve, formed with time intervals as abscissæ and daily concentrations of antibody per cc as ordinates, multiplied by the plasma volume. This new method of calculating available circulating antibody has also been applied successfully to the data of Glenny and Hopkins<sup>3</sup> on diphtheric antitoxin and hence may be of general immunological interest.

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<sup>3</sup> Smith, M. L., and Price, S. A., *J. Path. and Bact.*, 1938, **47**, 361.



extended from 2 to 4 weeks. The maximal increments in titer after 1000 units per kg of antitoxin, varied from 6 to 10 units. After 2000 units, maximal titers varied from 15 to 25 units. In monkeys injected with similar doses, phase A lasted 1 to 4 days, phase B, 3 to 6 days, phase C, from 4 to 5 weeks. The maximal increments were much higher than in humans—the titers ranging from 15 to 30 units after 1000 units of antitoxin and 35 to 50 after 2000 units per kg. It must be emphasized that the natural titers of monkeys are higher than those of humans. In the latter they were less than 1 unit whereas in monkeys they varied from less than 1 to 7 in this series, but may reach as high as 20 or more units per cc.<sup>6</sup>

In most instances, the serum titers reverted to their natural levels after elimination of the passively transferred antibody.

Because of individual variations in maximal serum titers and in the time of elimination of the antitoxin, it became necessary to develop a method of indicating the total available circulating units of antitoxin during a given period of time as the result of the injection of a given dose. By plotting on standard graph paper, concentrations of *alpha* antistaphylolysin per cc of serum as ordinates and time, in days after injection, as abscissæ, and integrating these curves, the areas underneath represent the summation of concentrations of antibody that have left their storehouse in the reticulo-endothelial system<sup>7</sup> and appeared in the blood stream during the period of observation. The areas may be conveniently determined by weighing the paper under the curve, counting the units squares or by use of a planimeter.

When 5 monkeys were each injected with 1000 units per kg, the average concentration of available antibody during 9 days was 113.5 units per cc (Table I). In 4 other monkeys similarly injected with twice the dose per kg, the average was double or 233.8. In human subjects the values for similar dosage and time-intervals were approximately 43 and 93 respectively, the ratio being again approximately 1:2. Multiplying these values by the weights and the factor 54, one obtains the number of units of antitoxin appearing in the total plasma-volume.<sup>8</sup> Thus in persons receiving 1000 units per kg the total available circulating units in the course of 9 days was 141,550, whereas in 2 others receiving approximately twice this dose per kg the total was about twice as large, or 288,115. The corresponding average values for monkeys are 19,160 and 32,620. In all instances,

<sup>6</sup> Weiss, C, *J. Immunol.*, 1939, **37**, 185.

<sup>7</sup> Sabin, F. R., *J. Exp. Med.*, 1939, **70**, 67.

<sup>8</sup> Brown, G. E., and Keith, H. Z., quoted in Peters, J. P., and van Slyke, D. D., *Quantitative Clinical Chemistry* Williams and Wilkins, Baltimore, 1935, **1**, 725.

cord, and the second and third from the jugular vein or the anterior fontanel. The serum volume index is determined as follows:

Five cc of blood is allowed to stand at room temperature in a graduated tube for 4 hours, at the end of which time the serum volume expressed from the clot is read. A red blood cell count is also made. In normal individuals the serum expressed is 50% of the blood volume. The index equals the serum volume of the patient studied over half the volume of blood withdrawn for the test, a correction is made for anemia if it exists. One (1) is the standard of normal. The highest index at which a jaundiced patient has bled is .71.

The maternal values (Table I) in these 11 patients are rather lower than are normally found, the decreases perhaps being due to the loss of blood at delivery. Some confirmation of this supposition is furnished by the almost uniform rise in the second and third maternal indices. The infant indices parallel the observations made by other tests in all respects. They are considerably lower than the maternal indices, the lowered values found immediately after delivery are still further decreased on the third day of life, and there is a uniform rise, usually with a return to the initial values or higher values, on the fifth day of life. The values reported are paralleled by values in other, less complete, maternal and fetal studies by the serum volume index and also by values in studies by the bedside test<sup>6</sup> to be reported elsewhere.

In 4 instances the fetal indices immediately after birth fell within the range at which bleeding would be expected in the adult, and 9 fetal indices on the third day were well below the safe level. Bleeding did not occur in any of these children, nor does it seem to have occurred in other instances<sup>1, 2</sup> in which the prothrombin values were well

TABLE I.  
Studies by the Serum Volume Index (for the Hemorrhagic Diathesis) in Parturient Women and Newborn Children

Mother			Child		
At delivery	Third day	Fifth day	At delivery	Third day	Fifth day
.76	.67	.87	.76	.77	.82
.87	.91	.91	.70	.65	.64
.87	.88	.80	.86	.57	.73
.80	.86	.88	.71	.65	.72
.88	.94	.91	.66	.60	.63
.71	.80	.80	.65	.61	.81
.81	.94		.96	.47	
.75	.80	.81	.99	.88	.98
.83	.81	.80	.71	.62	.73
.87	.89	.92	.89	.51	.75
.89	.89	.92	.69	.61	.57

## Serum Volume Index Studies on Newborn Children

HENRY LEIDENHEIMER, JR., AND FREDERICK FITZHERBERT BOYCE.  
(Introduced by Richard Ashman)

*From the Department of Obstetrics and Gynecology, School of Medicine, and Department of Surgery, Graduate School of Medicine, Louisiana State University*

Although reports differ in detail,<sup>1-5</sup> it has been fully established that a hypoprothrombinemia occurs in infants shortly after birth, that its duration is brief, and that normal values are usually reestablished by the fifth day of life. That this deficiency is related to hemorrhagic disease of the newborn is suggested by the fact that the hemorrhagic state coincides with the period of hypoprothrombinemia and by its prompt correction by vitamin K therapy.<sup>2-6</sup>

Previous studies have been made by the prothrombin titration technic of Quick,<sup>7</sup> the two-stage titration technic of Warner, Brinkhous and Smith,<sup>8</sup> the bedside test of Ziffren, Owen, Hoffman and Smith,<sup>9</sup> and the coagulation deficiency test of Dam and Glavind.<sup>10</sup> The studies herewith reported were made by the serum volume test,<sup>11, 12</sup> which was devised to predict the hemorrhagic diathesis in jaundice and has been used with entire satisfaction for this purpose for the last 5 years.

The studies were made on 6 white and 5 colored mothers and their full-term babies. The maternal blood was secured from the antecubital vein. The first samples in the children were secured from the

<sup>1</sup> Brinkhous, K. M., Smith, H. P., and Warner, E. D., *Am. J. M. Sc.*, 1937, 103, 475.

<sup>2</sup> Quick, A. J., and Grossman, A. M., *Proc. Soc. Exp. Biol. and Med.*, 1939, 41, 227.

<sup>3</sup> Hellman, L. M., and Shettles, L. B., *Bull. Johns Hopkins Hosp.*, 1939, 65, 138.

<sup>4</sup> Waddell, W. W., Jr., and Guerry, DuP., *J. A. M. A.*, 1939, 112, 2250.

<sup>5</sup> Shettles, L. B., Delfs, E., and Hellman, L. M., *Bull. Johns Hopkins Hosp.*, 1939, 65, 419.

<sup>6</sup> Dam, H., Tage Hansen, E., and Plum, P., *Lancet*, 1939, 2, 1157.

<sup>7</sup> Quick, A. J., Stanley Brown, M., and Bancroft, F. W., *Am. J. M. Sc.*, 1936, 100, 501.

<sup>8</sup> Warner, E. D., Brinkhous, K. M., and Smith, H. P., *Am. J. Physiol.*, 1936, 114, 607.

<sup>9</sup> Ziffren, S. E., Owen, C. A., Hoffman, G. R., and Smith, H. P., *Proc. Soc. Exp. Biol. and Med.*, 1939, 40, 595.

<sup>10</sup> Dam, H., and Glavind, J., *Acta med. Scandinav.*, 1938, 96, 108.

<sup>11</sup> Boyce, F. F., and McFetridge, E. M., *J. Lab. and Clin. Med.*, 1937, 23, 202.

<sup>12</sup> Boyce, F. F., and McFetridge, E. M., *New Orleans M. and S. J.*, 1939, 91, 357.

cavity of fleas collected from a dog having a heavy infection. On other dogs harboring *Dirofilaria immitis*, *Ctenocephalides felis* and *Pulex irritans* were found similarly infected. The female fleas of the 3 species were much more frequently infected and were more heavily infected with all the larval stages of the parasite than were the males. From some dogs infected with heartworm as many as 100% of the female fleas examined contained one or more infective-stage larvae and many of the earlier stages of development. The males harbored these stages only in small numbers and rarely contained the infective stage larvae.

Fleas of these 3 species collected from uninfected dogs have been experimentally infected in the laboratory in New Orleans and the extra-mammalian phase of the life cycle of *Dirofilaria immitis* has been completed in 10 such experiments undertaken. Sections of the infected fleas showed that the parasite in the typical microfilarial stage as found in the dog's blood, had entered the ventral part of the hemocelic cavity in the region occupied by the fat bodies and had there begun its development. The metamorphosis of the parasite from the microfilaria to the infective stage larva followed closely that found in various species of mosquitoes infected here<sup>3</sup> and by other workers elsewhere. The microfilaria developed into a shortened, so-called sausage larva, identical with that found in experimentally infected mosquitoes. By gradual elongation of the body and development of the internal structures this sausage larva became the infective stage larva which was morphologically identical with that developing in mosquitoes, although the time required for complete development in the flea was much shorter than in any mosquito worked with here or as yet reported in the literature. Thus far the minimum time required for development in the flea, from the microfilarial to infective stage, was 120 hours as compared with 240 hours in species of mosquitoes experimentally infected here. In addition, the writer's observations indicate that the microfilaria did not begin its development in the Malpighian tubules as Breinl had stated for fleas and as occurs in mosquitoes, but rather in the hemocelic cavity.

In experiments conducted with fleas during the months of June and July it was found that the infective stage larva of *Dirofilaria immitis* appeared in 120 hours as compared with 216 hours in the same species of flea in experiments conducted in December and January. Observations as yet incomplete seem to indicate that during

<sup>3</sup> Hinman, E. H., *Am J Trop Med*, 1934, 15, 371

<sup>4</sup> Writer's observations

below normal Whether children bleed at a lower level than adults, so that a fresh set of standards must be devised for them, it is not now possible to say A partial explanation of the lower maternal and fetal values may be that all of these tests were run during the winter, when the intake of foodstuffs containing vitamin K is less than in the summer months<sup>5</sup>

*Summary* A serial study of 11 parturient women and their infants by the serum volume index confirmed the findings of others, by other tests, that a definite tendency to hemorrhage exists in infants for a short time shortly after birth

## 11227 P

### Fleas as Acceptable Intermediate Hosts of the Dog Heartworm, *Dirofilaria immitis*

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The method of natural transmission of the dog heartworm, *Dirofilaria immitis*, is still incompletely elucidated The larvae of this parasite have been shown to develop in many species of mosquitoes and, thus, mosquitoes have been believed to be incriminated in the natural transmission, but substantial proof of this is lacking The possibility that other blood-sucking arthropods serve as transmitters was overlooked until 1921, when Breml<sup>1</sup> reported finding the microfilarial embryos of *Dirofilaria immitis* in the fleas *Ctenocephalides canis* and *Ctenocephalides felis*, collected from heartworm infected dogs in Australia He also observed the advanced stages of development of this parasite in the Malpighian tubules of the fleas and the infective stage larvae in the hemocelic cavity Recently, Brown<sup>2</sup> has observed the microfilariae and partially developed larvae of this parasite in *Ctenocephalides canis* collected from heartworm-infected dogs in the eastern United States

In November of 1938, the writer, in examining dogs for heartworm infection in New Orleans, observed the typical microfilariae of *Dirofilaria immitis* in *Ctenocephalides canis* The early developmental stages and the infective larvae were also seen in the hemocelic

<sup>1</sup> Breml, A., *Ann Trop Med*, 1921, 14, 389

<sup>2</sup> Brown, H. W., *The North Amer Veterinarian*, 1939 20, No 1

thyroid-fed rats by feeding large amounts of yeast. It is also known that the dietary requirements for vitamin B<sub>1</sub> and the B<sub>2</sub> complex are above normal during experimental hyperthyroidism in rats.<sup>7</sup> Since yeast can maintain the liver glycogen of thyroid-fed rats we decided to investigate any possible relationships between the liver function of hyperthyroid dogs and the yeast content of the diet. No studies of the liver function of hyperthyroid animals are reported in the literature.

**Methods** Throughout the experiment the dogs received a modified form of Cowgill's casein diet No. III,<sup>8</sup> in which 21% lard was used, and the butter was replaced by 4% of cod liver oil, so that a known amount of vitamins A and D were added to the diet. The dogs used were all full-grown males weighing between 9 and 17 kg. The dogs were fed a stock diet of Purina checkers *ad libitum*. Two weeks before the thyroid feeding was started the dogs were placed on the modified Cowgill diet. The dogs were allowed to eat as much as they wanted for a 3-hour period each day. Each dog received a daily supplement of yeast No. 17800 in the proportion of 2 International units of vitamin B<sub>1</sub> per pound of body weight. The yeast contained 23 I U. of vitamin B<sub>1</sub> per gram and 20 Sherman-Borquin units of vitamin G (flavin) per gram.\* This makes the diet normal in all respects. The thyroid used was Lilly's desiccated thyroid gland USP.

The bromsulphalein method<sup>9</sup> was used to determine the liver function of the dogs. Five mg of dye per kg of body weight was injected intravenously into the jugular vein of unanesthetized trained dogs, and 30 minutes later 10 cc of blood was withdrawn under oil from the opposite jugular and the concentration of dye remaining in the serum determined. In the standards 4 mg of bromsulphalein per 100 cc of dilute NaOH was used as 100%.

**Results** The 2 control dogs, receiving only Cowgill's diet and the daily yeast supplement, showed normal liver functions throughout the experiment. The dye retention in the serum of the controls at the end of 30 minutes was always less than 10%, generally being between 2 and 8%. Any retention of dye above 15% in the thyroid-fed animals was therefore considered abnormal and definite evidence of impaired liver function.

<sup>7</sup> Drill, V. A., and Sherwood, C. R., *Am. J. Physiol.* 1938, **124**, 693.

<sup>8</sup> Cowgill, G. R., *Am. J. Physiol.*, 1928, **83**, 45.

\* The authors wish to thank Dr. H. W. Rhodelamel of Eli Lilly and Co. for supplying the large amount of desiccated thyroid gland that was required, and also Dr. C. N. Frey of the Fleischmann Laboratories for supplying the analysed yeast.

<sup>9</sup> Rosenthal, S. M., and White, E. C., *J. Am. Med. Assn.*, 1925, **84**, 1112.

the warmer season of the year the time required for development is shorter than during the colder season. With decreasing temperature the time required for development increases and *vice versa*.

In summary, the fleas, *Ctenocephalides canis*, *C. felis* and *Pulex irritans*, collected from dogs harboring *Dirofilaria immitis*, have been found naturally infected with the microfilarial and larval stages of this parasite. The female fleas seemed much more susceptible to the infection than the males. All 3 species of fleas have been experimentally infected and in all 3 the extra-mammalian phase of the life cycle has been completed. The time required for this development in fleas was 120 hours in warm weather and 216 hours in cold weather. This phase of the life cycle was more rapidly achieved in fleas than in mosquitoes. It appears that both biologically and epidemiologically fleas are more suitable intermediate hosts of *Dirofilaria immitis* than had been previously supposed.

## 11228 P

## Hyperthyroidism and Liver Function in Relation to B Vitamins

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From the Biological Laboratory, Princeton University, Princeton, N. J.

Many authors have reported that the liver becomes altered both structurally<sup>1</sup> and functionally<sup>2</sup> during hyperthyroidism. One of the most marked changes in the liver during the experimental and clinical hyperthyroid states is a depletion of the liver glycogen.<sup>1, 3</sup> A high carbohydrate diet is usually given to hyperthyroid patients in an effort to promote glycogen storage in the liver.

Abelin<sup>4</sup> and Abelin, *et al.*,<sup>5</sup> showed that large amounts of casein egg yolk, or yeast would lessen the liver damage of the thyroid-fed animals, and Drill<sup>6</sup> was able to prevent a fall in the liver glycogen of

<sup>1</sup> cf. Frazier, C. H., and Brown, R. B., *Trans. Am. Assn. for Study of Goiter*, 1935.

<sup>2</sup> Maddock, W. G., Pedersen, S., and Coller, F. A., *J. Am. Med. Assn.* 1937, 100, 2130 cf. reference<sup>1</sup>.

<sup>3</sup> John, H. J., *J. Am. Med. Assn.*, 1932, 99, 620.

<sup>4</sup> Abelin, I., *Biochem. Z.*, 1930, 228, 165.

<sup>5</sup> Abelin, I., Knoedel, M., and Spiechlin, W., *Biochem. Z.*, 1930, 228, 189.

<sup>6</sup> Drill, V. A., *J. Nutrition*, 1937, 14, 355.

results are best obtained with a dosage of 0.6 g of thyroid gland per kg of body weight. It is known that the amount of vitamin B<sub>1</sub> in rat tissues is reduced by thyroid feeding,<sup>10, 11</sup> and that the thyroid-fed rat also requires more of the vitamin B<sub>2</sub> complex than is supplied in a normal diet. We have found this same dietary relationship to be present in the dog.<sup>12</sup> This indicates that the abnormal liver functions in the thyroid-fed dogs are related to an increased requirement for some of the B vitamins, with a probable loss in body stores, as judged by the loss of appetite. This will be reported later in greater detail. A subnormal amount of the B vitamins in the diet may be at least partially responsible for the abnormal liver function that is observed in human hyperthyroidism.

The results of these experiments do not mean that the B vitamins are the only factors related to the production of abnormal liver function in hyperthyroid animals. However, this is the first causal relationship to be established for the production of abnormal liver function in hyperthyroidism. It is not yet known if a large amount of yeast in the diet can maintain a normal liver function in thyroid-fed dogs over a long period of time. Further experiments are in progress.

**Conclusions** 1 Using a standard diet, with yeast of a known vitamin content, the liver functions of dogs was studied at two levels of thyroid feeding. 2 The production of the abnormal liver function in hyperthyroid dogs bears a causal relationship to the yeast in the diet.

11229

### X-radiation and Growth Substances as Affecting Plant Primordial Tissues

VIRGINIA C. IRVINE\* (Introduced by Francis Ramaley)

*From the Department of Biology, University of Colorado, Boulder, Colo*

Since little attention has been given to the primordial regions of plants as influenced by X-radiation and chemical growth-promoting substances the author has carried out experiments to determine effects of these agencies on growing points. Snow<sup>1</sup> observed that hetero-

<sup>10</sup> Drill, V. A., *Am. J. Physiol.*, 1938, **122**, 486

<sup>11</sup> Peters, R. A., and Rossiter, R. J., *Biochem. J.*, 1939, **33**, 1140

<sup>12</sup> Drill, V. A., unpublished work.

\* University Scholar in Biology

<sup>1</sup> Snow, M. and R., *New Phytologist*, 1937, **36**, 1



*Dogs fed 0.6 gm thyroid gland/kg of body weight* Two dogs (Nos 3 and 11) were fed this dosage. Dog No 3 showed a normal liver function up to the 42nd day of thyroid feeding. On the 42nd day the yeast was reduced to one-half of its amount in the diet and 24 hours later the liver function showed 25% of dye retention. On the 44th day the yeast was removed from the diet and 24 hours later the test showed 150% retention of dye. More than 100% retention of dye is due to the fact that the colorimeter standards developed originally for the injection of 2 mg of dye per kg of body weight, have been retained even though a 5 mg dose is used. It is difficult to differentiate the color with higher standards.<sup>2</sup> The dye retention then dropped and remained at an average of 50% during the next 10 days. Dog No 11 showed a marked drop in appetite after the 37th day of thyroid feeding. This indicated that the vitamin stores, in particular vitamin B<sub>1</sub>, of the body were lowered and that now more vitamins were being metabolized than were being supplied in the normal diet. A liver function on the 41st day gave 45% retention.

*Dogs fed 0.4 g of thyroid gland/kg of body weight* Dogs Nos 2, 9, and 10 received this dosage. Dog No 2 gave normal liver functions up to the 49th day of thyroid feeding. A test on the 59th day showed 25% retention. The yeast was then removed from the diet and 24 hours later 125% retention of dye was obtained. This dropped and remained between 25-40% during the next 10 days. Dog No 10 showed similar results. Dog No 9 gained a considerable amount of weight after the experiment had started, so that on his new weight basis he was receiving less than 0.4 g of thyroid/kg of body weight. His food intake was still above normal, and was not dropping, indicating that no depletion of the body stores of vitamins had occurred. On the 57th day his liver function was still normal, and no fall in appetite was observed. The yeast was removed from the diet on the 57th day and 24 hours later only 20% of dye retention was observed, which remained at this low level during the next 5 days. This shows that without vitamin depletion, as shown by the high food intake, that only a slightly abnormal liver function was obtained when the yeast was removed.

*Discussion* The results indicate a definite relationship between the yeast content of the diet and the liver function of thyroid-fed animals.

Some of the dogs maintained normal liver functions for 40-50 days on the above diet while receiving thyroid gland, and then showed a marked abnormal liver function as soon as the yeast was removed. In the others the liver function became abnormal, without removing the yeast from the diet, in about 40 to 50 days. These

results are best obtained with a dosage of 0.6 g of thyroid gland per kg of body weight. It is known that the amount of vitamin B<sub>1</sub> in rat tissues is reduced by thyroid feeding,<sup>10, 11</sup> and that the thyroid-fed rat also requires more of the vitamin B<sub>2</sub> complex than is supplied in a normal diet.<sup>7</sup> We have found this same dietary relationship to be present in the dog.<sup>12</sup> This indicates that the abnormal liver functions in the thyroid-fed dogs are related to an increased requirement for some of the B vitamins, with a probable loss in body stores, as judged by the loss of appetite. This will be reported later in greater detail. A subnormal amount of the B vitamins in the diet may be at least partially responsible for the abnormal liver function that is observed in human hyperthyroidism.

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<sup>12</sup> Drill, V. A., unpublished work.

\* University Scholar in Biology.

<sup>1</sup> Snow, M. and R., *New Phytologist*, 1937, **30**, 1.

auxin applied to the growing regions of certain plants caused union of leaf primordia, and Bausor<sup>2</sup> describes the development of root primordia on stems, petioles, and apical meristem when these parts are treated with beta-naphthoxyacetic acid

In the present study, seedlings of sunflower (*Helianthus annuus*), zinnia (*Zinnia elegans*), and tomato (*Lycopersicum esculentum*) were given moderate X-ray doses (2300 to 2500 r-units for sunflower and zinnia and up to 3000 r-units for tomato) Plants were stunted, and showed rough, warty, and abnormally-shaped leaves, with fasciation of leaves and of stems and frequent fusion of leaf primordia, as previously reported by Johnson<sup>3</sup> Often, a few weeks after treatment, the meristematic region was divided into 2 or 3 parts, each with a separate group of primordial leaves, showing that the stem was about to branch Indole-3-acetic acid (0.5 to 1.0%) applied in lanolin paste to seedlings of the above-named species caused increased growth, and with higher concentrations (2%), a fusion of leaves The meristematic area either resembles that of untreated controls, or various leaf primordia may become joined, or else displaced from normal position Colchicine (0.5%) applied also in lanolin paste, was found to retard growth, and it induced development of rough, warty, and misshapen leaves just as X-radiation does, while short

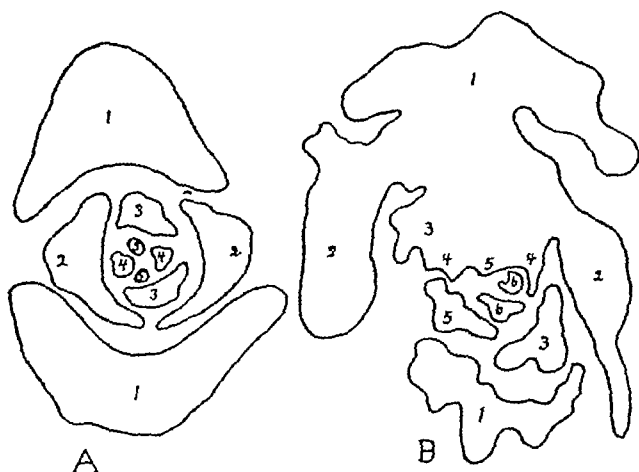


FIG 1.

Cross sections through primordial regions of sunflower stem A, control, B, 3 weeks after treatment with beta naphthoxyacetic acid Note the distorted and fused primordia (Numbers on the diagrams indicate leaf pairs)

<sup>2</sup> Bausor, S C, *Am J Botany*, 1939, **26**, 733

<sup>3</sup> Johnson, E L., *Plant Physiology*, 1936, **11**, 319

TABLE I  
Effects of 2% beta naphthoxyacetic acid upon sunflower, zinnia, and tomato  
(Results given as percentage of plants affected.)

Effects noted	Sunflower	Zinnia	Tomato
Stunted growth	100	100	97
Branching	45	53	25
Distorted leaves	100	100	100
Disturbed phyllotaxy	84	88	86
Fusion of leaves or of leaf segments	89	90	88
Fusion of primordia	75	89	83
Displaced primordia	70	74	70
Splitting of apex	25	33	30
Fasciation	30	34	30

malformed leaf primordia are also produced. The effects of alpha-naphthoxyacetic acid and of beta-naphthoxyacetic acid are similar to the effects of colchicine, but usually less pronounced. The beta form is more potent than the alpha, and since little has been published concerning its action on plants a figure and table are here introduced to describe it.

In general, beta-naphthoxyacetic acid and the other chemicals used in this study are found to produce results upon primordial plant tissues similar to the effects of X-radiation.

11230 P

### Droplet Infection of Air High-speed Photography of Droplet Production by Sneezing \*

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The question of infection of air by droplets given off in coughing and sneezing has received considerable attention during the last few years. In particular, investigation has been directed towards determining the rôle of the air-borne droplet nuclei which result from the evaporation of droplets proper. The bacteriological and epidemiological aspects have recently been discussed by Wells, Wells, and Mudd.<sup>1</sup> Experimentally, little is known of certain of the characteristics of such droplets—their number, size, velocity, settling rate, and rate of

\* Contribution No. 163 from the Department of Biology and Public Health.

<sup>1</sup> Wells, W F, Wells, W W, and Mudd, S, *Am J Pub Health*, 1939, **29**, 863

evaporation—although these factors are concerned in their dissemination and the production of droplet nuclei. Wells<sup>2</sup> has calculated theoretical settling times and distances of fall before evaporation, for plain water droplets of various sizes suspended in still air of different humidities. These figures are only roughly applicable to actual conditions of air infection, since mouth spray and naso-pharynx droplets will contain some dissolved solids or be mucus-like, and the air will not be still. Rooks<sup>3</sup> has shown experimentally that droplet size is an important factor in nasal filtration, although he knew only the theoretical differences in average size of the droplets with which he worked. Weyrauch and Rzymkowski<sup>4</sup> have photographed the tracks of moving droplets given off in sneezing and talking. The paths shown indicate that most of the actual droplets travel only relatively short horizontal distances.

We have been able, by means of high-speed photography, to "stop" the motion of droplets given off in coughing and sneezing, thereby permitting measurements of droplet size, velocity, etc. The technique utilizes the light source and control instruments developed by Edgerton, *et al.*,<sup>5</sup> for stroboscopic illumination and high-speed photography. The light source, which is placed at one side of the subject's face consists of a 9-inch specular reflector with a spiral argon-filled tube through which a 56 microfarad condenser (charged to 2500 volts) discharges. An intense flash of short duration is produced, illuminating the droplets with a dark-field effect so that they stand out sharply even in daylight. The photographically effective duration of flash (exposure time) may be adjusted to the velocity of the particles whose motion is to be stopped. An electrical contact on the camera shutter synchronizes the flash with the shutter motion.

Figure 1 shows the result of a sneeze at the end of the 'down-stroke' of the head. Most of the droplets have already been expelled of these, some 4600 may be counted which were in the focal plane of the camera. This photograph was taken with an ordinary camera on 9 x 12 cm film with an *f*11 aperture and an exposure of 1/15 000 of a second. In spite of this short exposure below the nose may be seen the paths of droplets which moved during that time. (Electrical characteristics of the light source account for the 'reversed head and tail' appearance of these particle paths.) Calcula-

<sup>2</sup> Wells, W. F., *Am J Hyg*, 1934, 20, 611.

<sup>3</sup> Rooks, R., *Am J Hyg*, 1939, 30, 7.

<sup>4</sup> Weyrauch, F., and Rzymkowski, J., *Z f Hyg u Infektionskr*, 1938, 120, 444.

<sup>5</sup> Edgerton, H. E., Gemeshausen, K. J., and Grier, H. E., *J Appl Physics*,



FIG. 1

Droplets resulting from a sneeze. Instantaneous photograph (exposure  $1/15,000$  of a second) taken at the end of the "down stroke" of the head. Note tracks of moving particles beneath the nose and the string of saliva issuing from the mouth.

tion shows the fastest of these droplets to be moving at a rate of over 100 feet per second. Such a velocity in dry air would result in nearly instantaneous evaporation producing droplet nuclei. The significance of the velocity of expulsion in relation to evaporation is perhaps greater than has been appreciated; it would appear to be a more important factor than settling velocity.

As regards droplet size, optical considerations indicate that only those in sharp focus give photographic images approximating the *true* particle size. The range of *apparent* diameter of the great majority of sneeze droplets before appreciable evaporation occurs has been determined from photographic enlargements to be  $1/10$  to  $2$  mm, which figures are probably maximum rather than minimum diameters.

We have observed that the involuntary closing of the mouth near the end of a sneeze tends to produce more and smaller droplets, which probably come largely from the saliva in the front of the mouth. Also, the number of droplets issuing from the nose is usually insignificant compared with the number expelled from the mouth. These observa-

tions may be important in relation to infectivity, because of the differences in the microbic flora of the two regions

Problems of rates of and distances to evaporation, and of minimum droplet size are being investigated

### 11231 P

#### Effect of Repeated Injections of Cobra Venom on Blood Chemistry and Morphology

DAVID I. MACHT, SOLOMON SHERMAN AND DOROTHY J. BROOKS

*From the Pharmacological Research Laboratory, Hynson, Westcott & Dunning, Inc., Baltimore Md*

Large doses of cobra venom were repeatedly injected in rabbits for periods varying from 2 to 21 weeks and quantitative studies were made on the morphology and chemistry of their blood. The venom solution was usually injected 5 times a week and the dosage administered on injection varied from 5 to 10 mouse units. Inasmuch as 5 mouse units constitute the usual therapeutic dose of cobra venom for human beings and 10 mouse units are employed only in more refractory cases, the dosages given rabbits in the present study were manifestly enormous. The results obtained from 10 rabbits are reported in this investigation. Three of the rabbits were used as controls and received no cobra venom while the other 7 received the drug partly by the intravenous and partly by the intramuscular route. Rabbits of different weight were purposely selected at the beginning of the research but all were kept under the same conditions and fed with Purina Chow supplemented with fresh green vegetables. The cobra venom solution (H. W. & D.) employed was prepared in these laboratories and assayed by the authors on white mice by methods described elsewhere.<sup>1</sup> This solution is the same as that employed by various authors in clinical studies published in different journals.<sup>2-6</sup> Crude cobra venom consists largely of neurotoxin and contains relatively small quantities of cytotoxic and proteolytic constituents in striking

<sup>1</sup> Macht, *Proc. Nat. Acad. Sc.*, 1936, **22**, 61

<sup>2</sup> Macht, *Med. Rec.*, 1936, **144**, 537

<sup>3</sup> Macht, *Ann. Int. Med.*, 1938, **11**, 1824

<sup>4</sup> Gayle and Williams, *Southern M. J.*, 1938, **31**, 188

<sup>5</sup> Rutherford, *New England J. Med.* 1939, **221**, 408

<sup>6</sup> Black, *Southern M. J.* 1940,

contrast to the crude venom of the *Viperidae* (*Crotalus*, *Bothrops*, *Akistrodon*, and Russell's viper), which are rich in cytotoxic and blood-affecting elements but contain very small amounts of the neurotoxic substance. In the preparation of the cobra venom solution employed by the authors and in the process of sterilizing it, the proteins of the crude substance are almost entirely removed and the cytotoxic and hemolytic constituents are eliminated by at least 50%. The resultant solution of cobra venom therefore consists largely of the neurotoxin which is quite stable and is responsible for the analgesic and other therapeutic properties of cobra venom. This neurotoxin, according to the latest chemical work on the subject, is not a protein but resembles the glucosides in its structure.<sup>8</sup>

In the present study the authors have determined the absolute number of erythrocytes and leukocytes, the differential leukocyte count and the percentage of hemoglobin in the blood. Quantitative biochemical determinations were also made on blood urea, blood urea nitrogen and blood sugar by the usual standard clinical methods (Folin). The results obtained are exhibited in Table I, which indicates the average weight of the animals, the total amount of venom injected in each and the period over which the drug was administered, in addition to the data obtained from the morphological and biochemical examination of the blood. Kidney and liver function tests were also made in these rabbits but are not mentioned in the table as they have already been discussed elsewhere in these PROCEEDINGS.<sup>9</sup> It will be seen that the data set forth in the tables reveals that no specific change occurred in the blood of the treated rabbits as compared with the normal controls and with each other. Certainly no characteristic injuries or pathological effects were noted in the blood examinations. The considerable anemia and high eosinophile count obtained in Rabbit No. 1 were due to the parasitic fungous infection of the ears frequently encountered in laboratory rabbits. The total amount of cobra venom injected in the rabbits varied from 45 to 990 mouse units. None of the rabbits treated with cobra venom revealed any difference in duration of coagulation time. The weight of the animals determined at the end of a period of experimentation differed but slightly from that registered at the beginning of the research. The young rabbits of course, increased in weight as they grew older but the increment in this case was the same as in the normal controls. Further researches along the above-mentioned

<sup>8</sup> Noc, *Ann Inst Pasteur*, 1904, **18**, 357.

<sup>9</sup> Michael and Bode, *Naturwissenschaften* 1938, **20**, 298.

<sup>9</sup> Maclit and Brooks, *Proc Soc Exp Biol and Med*, 1939, **41**, 418.



TABLE I  
Effect of Cobra Venom on Morphology and Chemistry of the Blood of Rabbits

Rabbit No	Avg kg wt of rabbit	No of weeks rabbit inf	Total No mouse cobra venom inf	Blood urea	Blood urea N	% of blood sugar	Red blood corpuscles	% of hemo globin	Leuko cyt count	Differential Leukocyte Count						Neutro phic myelo cytes
										Small lympho cytes	Large lympho cytes	Large mono nuclears	Poly morpho nuclears	Dosino philes	Baso philes	
1	3.65	13	990	73.5	25.0	0.119	4,176,000	58	14,270	27	7	15	15	32	4	—
2	2.68	12	630	49.2	21.0	0.103	5,600,000	71	18,200	55	14	2	23	6	—	—
3	2.30	3	90	49.2	23.0	0.147	5,632,000	81	17,130	59	13	7	16	1	2	2
4	2.24	21	850	40.0	18.7	0.153	5,368,000	85	8,430	50	16	7	24	2	—	1
5	1.00	Control	0	49.2	23.0	0.111	5,056,000	75	9,600	64	6	3	25	1	—	1
6	4.52	21	60	61.0	28.5	0.107	5,328,000	78	8,800	39	4	21	33	—	—	1
7	2.00	Control	0	78.7	18.1	0.162	5,954,000	95	10,550	68	5	4	18	3	1	1
8	1.95	7	265	45.8	21.4	0.100	5,840,000	96	14,200	53	11	7	22	3	3	1
9	4.00	Control	0	45.8	21.4	0.111	4,080,000	95	11,200	65	6	5	21	3	—	—
10	3.00	2	45	49.2	23.0	0.133	4,144,000	76	9,270	52	8	6	26	4	—	—

lines are in progress. It is interesting to note that the findings set forth in the present paper regarding rabbits injected with large doses of cobra venom over long periods of time agree with the clinical observations of Steinbrocker, McEachern, La Motta and Brooks<sup>10</sup> in their study on human subjects with material supplied by the senior author of the present paper. These findings agree also with the undetailed report recently published concerning the work of French investigators who found that no change was produced in the blood sugar level of rabbits but that a rise in blood sugar of guinea pigs was effected by injections of a cobra venom preparation, regarding which details are not given.<sup>11</sup>

*Summary.* Large quantities of cobra venom were injected in a series of rabbits for periods varying from 2 to 21 weeks. Morphological and biochemical studies on the blood revealed no striking pathological change and no specific effect on the blood picture of the animals as compared with normal controls.

## 11232 P

## Toxicity for Dogs of a Bactericidal Substance Derived from a Soil Bacillus

COLIN M. MACLEOD, GEORGE S. MIRICK AND EDWARD C. CURNEN  
(Introduced by Rene J. Dubos)

*From the Hospital of The Rockefeller Institute for Medical Research, New York City*

Dubos<sup>1</sup> has described the isolation from a sporulating soil bacillus of an agent which exerts a powerful bactericidal effect *in vitro* upon Gram positive bacteria and which if injected intraperitoneally affords protection to white mice infected by the same route with pneumococci or hemolytic streptococci. Subsequently Dubos and Cattaneo<sup>2</sup> reported the results of studies with a protein-free preparation of the bactericidal agent which duplicated the earlier results obtained with the crude protein-containing extract. The present report deals with the toxicity for dogs of a more highly purified protein-free preparation of the bactericidal agent injected by the intravenous route. The

<sup>10</sup> Steinbrocker, McEachern, La Motta and Brooks, *J. A. M. A.* 1940, **114**, 315.

<sup>11</sup> Foreign Letter from Paris, *J. I. M. I.*, 1940, **114**, 425.

<sup>1</sup> Dubos, R. I., *J. Exp. Med.* 1939, **70**, 1. Dubos, R. I., *Ibid.* 1939, **70**, 11.

<sup>2</sup> Dubos, R. I. and Cattaneo, C., *J. Exp. Med.* 1939, **70**, 249.

preparation used was active both *in vitro* and *in vivo* (mice) against Gram positive bacteria

Short-haired dogs weighing from 80 to 120 kg were chosen. Prior to the course of injections the animals were observed for from 1 to 2 weeks and only those showing a normal temperature curve, normal urinalysis and normal blood picture were used. Blood counts and urinalyses were performed daily during the course of injections and at intervals thereafter in the animals which survived. Temperature readings were taken at least twice daily and the animals were weighed frequently throughout the period of observation. Pathological study was made of all dogs which received injections of the bactericidal substance, those animals which did not die as a result of the injections were sacrificed at intervals following treatment. The bactericidal substance was dissolved in alcohol and dilutions of the alcoholic solution in 20 cc of a 5% glucose solution in redistilled water were used for intravenous injection. The doses ranged from 0.05 to 2.0 mg per kg (mg/kg) of body weight per day and injections were continued for 10 days in the dogs which survived (Fig 1).

Six of the 8 animals receiving 0.4 mg/kg or more per day died before the full course of injections was completed, the total dosage varying between 1.5 mg and 5 mg/kg. One dog receiving 0.5 mg/kg died on the 42nd day after the course of injections was begun and one animal which survived the 10 daily injections of 0.4 mg/kg was sacrificed on the 33rd day. None of the 5 dogs died which received a dosage of 0.3 mg/kg or less for 10 days. These were sacrificed

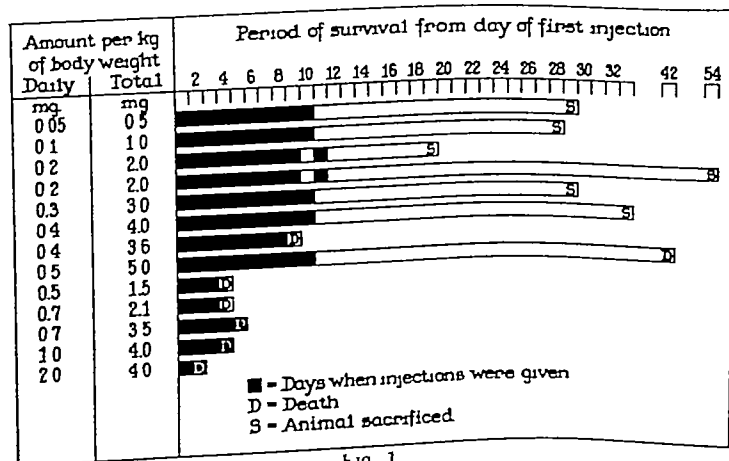


FIG 1  
Period of survival of dogs receiving varying amounts of the bactericidal substance

from 23 to 54 days after the course of injections was begun. All of the animals receiving 0.3 mg/kg or more per day showed well-marked toxic manifestations of either acute or chronic nature. In the animals receiving 0.2 mg/kg or less the evidence of toxicity was of minor degree.

The more prominent signs of toxicity were loss of weight associated with anorexia, fever which occurred particularly following the first few injections of the agent, progressive anemia, ascites, hematuria and the excretion of bile in the urine. In the animals which died acutely following injection of the larger doses (0.5 to 2.0 mg/kg) there was marked congestion present in the lungs and abdominal viscera with petechial hemorrhages in the heart, lungs and kidneys. The livers showed acute central necrosis associated with hemorrhage and dilatation of the sinusoids. Diffuse hemorrhage occurred in the spleen, with pronounced phagocytosis of red blood cells by the macrophages. In the kidneys hemorrhage was most marked in the glomeruli. In the animals which received daily 0.3 mg/kg or more and which did not die acutely, the changes in the organs were of a more chronic nature. The liver cells showed fatty degeneration which was most marked about the central veins. In these areas there was an increase in the reticular tissue, but cirrhotic changes were minimal. In 2 of the animals ascites was present. The only change noted in the organs of the animals receiving 0.2 mg/kg or less was a slight degree of fatty degeneration of the liver.

*Summary* A study has been made of the toxicity for dogs of a protein-free preparation of the bactericidal substance described by Dubos when injected by the intravenous route. Seven of the 8 animals which received 0.4 mg/kg or more daily died as a result of the injections and in 6 of these death occurred before the course of 10 daily injections was completed. All animals receiving 0.3 mg/kg or more showed well-marked acute or chronic changes in the liver, spleen, kidneys, heart and lungs. Animals which received daily 0.2 mg/kg or less for 10 days showed only minor evidence of toxicity.

preparation used was active both *in vitro* and *in vivo* (mice) against Gram positive bacteria

Short-haired dogs weighing from 80 to 120 kg were chosen. Prior to the course of injections the animals were observed for from 1 to 2 weeks and only those showing a normal temperature curve, normal urinalysis and normal blood picture were used. Blood counts and urinalyses were performed daily during the course of injections and at intervals thereafter in the animals which survived. Temperature readings were taken at least twice daily and the animals were weighed frequently throughout the period of observation. Pathological study was made of all dogs which received injections of the bactericidal substance, those animals which did not die as a result of the injections were sacrificed at intervals following treatment. The bactericidal substance was dissolved in alcohol and dilutions of the alcoholic solution in 20 cc of a 5% glucose solution in redistilled water were used for intravenous injection. The doses ranged from 0.05 to 2.0 mg per kg (mg/kg) of body weight per day and injections were continued for 10 days in the dogs which survived (Fig 1).

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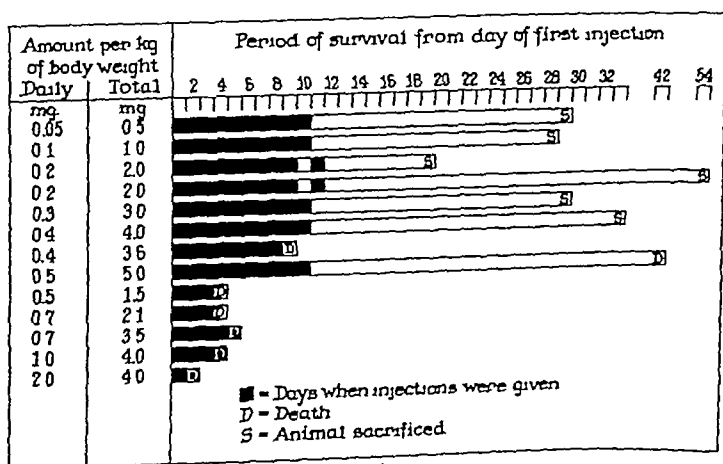


FIG 1

Period of survival of dogs receiving varying amounts of the bactericidal substance

ether anesthesia or placed under the influence of sedatives<sup>8</sup> More recently the property of excessive doses of insulin and metrazol to elicit shock or convulsions has been put to effective therapeutic use in the treatment of certain nervous disorders The precise mechanism of this form of therapy is still obscure Gross pathological changes in the brain which occur as the result of shock indicate severe structural damage to nerve cells<sup>9-11</sup> there is also evidence of depressed cerebral metabolism since hypoglycemic shock diminishes the oxygen utilization of brain tissue through the absence of available dextrose,<sup>12-15</sup> whereas metrazol convulsions achieve the same effect by decreasing the oxygen necessary for the combustion of this sugar<sup>16-20</sup> The resulting anoxemia may act by stimulating the sympathetic system<sup>21</sup> but it may also affect the brain directly by increasing cellular permeability<sup>22</sup> In view of what has been said above it was decided to determine whether the protracted administration of a powerful hypnotic like luminal or the production of systemic shock by means of insulin or metrazol had any effect on the course of poliomyelitis in the monkey

*Experiment I Effect of luminal depression* One Rhesus monkey, weighing about 2000 g received daily doses of 100 to 150 mg sodium luminal (sodium salt of phenyl-ethyl-malonyl urea) by the subcutaneous route beginning 3 days before intracerebral infection with poliomyelitis virus and continued for 7 days after infection An-

<sup>8</sup> Bronfenbrenner, J, and Weiss H, *J Exp Med* 1924 **30**, 517

<sup>9</sup> Schmid, H, *Ann Med psychol* 1936 **94** 658

<sup>10</sup> Weil A, Liebert E, and Heilbrunn G *Arch Neurol and Psych* 1938 **30**, 467

<sup>11</sup> Baker, A B *Arch Pathol* 1938 **26** 765

<sup>12</sup> Weil A and Liebert E *Arch Neurol and Psych* 1938 **30**, 1108

<sup>13</sup> Baker, A B *Am J Psych* 1939, **96**, 109

<sup>14</sup> Yarnet H, *Arch Neurol and Psych*, 1939, **42** 395

<sup>15</sup> Ferraro A and Jervis, G A *Am J Psych*, 1939, **96**, 103

<sup>16</sup> Holmes E G *Biochem J* 1930, **24**, 914, 1932, **26** 2010

<sup>17</sup> Gellhorn, E, *J Am Med Assn* 1938 **110** 1433

<sup>18</sup> Wortis S B, A T *State J Med*, 1938 **38** 1015

<sup>19</sup> Himwich H E, Bowman N M, Fazekas J F and Orenstein L L, *Proc Soc Exp Biol and Med* 1937, **37**, 359

<sup>20</sup> Low A A, Sonenthal I R, Blaurock M F, Kaplan M and Sherman I *Arch Neurol and Psych* 1938 **30** 717

<sup>21</sup> Himwich H E, Bowman N M, Wortis J and Fazekas J F, *J Am Med Assn* 1939, **112** 1572

<sup>22</sup> Gellhorn E *Arch Neurol and Psych* 1938 **40**, 125

<sup>23</sup> Spiegel E and Spiegel Adolf M, *Proc Soc Exp Biol and Med* 1939 **42**, 834

## Failure of Hypnotic and Convulsive Agents to Alter the Course of Experimental Poliomyelitis

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Little is known concerning the metabolism of the central nervous system in its relation to the factors which support or inhibit the propagation of neurotropic viruses. Yet it would seem that better knowledge of the chemical reactions which underlie the interaction between virus and substrate should materially contribute to our understanding of the infectious process itself. With the present analytical methods it is doubtful that a direct biochemical approach would yield any more concise data than are already on hand regarding such changes as disturbance in the operation of the oxidation-reduction potential of virus-infected nerve tissue<sup>1, 2</sup>. However an indirect attack of the problem may be feasible by studying the effect of comparatively crude alterations in the central nervous system—brought about by physiological or pharmacological methods—on the evolution of the characteristic virus lesion.

One recent attempt in this direction was made by Howe and Bodian<sup>3</sup>. By section of the axone these authors evidently succeeded in inducing an irreversible change in the metabolism of the nerve cell which rendered the affected territory relatively refractory to subsequent invasion by poliomyelitis virus. Certain drugs also cause a profound derangement in the metabolic function of the central nervous system particularly the narcotics and hypnotics which are capable of inhibiting the oxidation of substances essential in carbohydrate metabolism<sup>4</sup>. The possible usefulness of drugs of this type in modifying the action of neurotropic agents of disease has not yet been explored except for the observation that death from botulism can be delayed in guinea pigs when these animals are subjected to

<sup>1</sup> Brodie, M., and Wortis S. G. *Arch. Neurol. and Psych.* 1934 **32** 1159.

<sup>2</sup> Jungeblut C. W. and Feiner R. R. *J. Exp. Med.* 1937 **66** 479.

<sup>3</sup> Howe, H. A. and Bodian, D. *Proc. Soc. Exp. Biol. and Med.* 1939 **42** 41.

<sup>4</sup> Quastel J. H. and Wheatley A. H. M. *Biochem. J.* 1931 **25** 117. 1934 **28**

<sup>5</sup> Emerson G. A. *Proc. Soc. Exp. Biol. and Med.* 1935 **33** 171.

<sup>6</sup> Dameshek W., Myerson A. and Loman T. *Am. J. Psych.* 1934 **61** 11.

<sup>7</sup> Wortis S. B. *Am. J. Psych.* 1936 **63** 87.

hypoglycemic shocks during the entire course of the experiment. The results are listed in Table II. It appears from this table that the progress of the disease in the insulin-treated monkeys deviated in no significant way from that observed in the control animals. Likewise histological examination revealed no fundamental difference in the distribution or severity of the cord lesions between the treated and untreated group.

*Experiment III. Effect of metrazol shock.* One Rhesus monkey of 2000 g weight was injected intravenously with daily doses of 0.3 to 0.6 cc metrazol (1 cc = 100 mg of penta-methylene-tetrazol in 1% sodium phosphate solution) for a period of 4 days following intracerebral infection with poliomyelitis virus. Another monkey of similar weight received daily injections of the same dosage of the drug, beginning 5 days before poliomyelitic infection and discontinued thereafter. Two control monkeys infected with corresponding doses of virus (1 cc or 0.1 cc of a 5% virus suspension) but left untreated completed this experiment. The dose of metrazol was large enough to induce in both animals violent clonic-tonic convulsions which set in a few seconds after injection of the drug and lasted for from 5 to 10 minutes. The results are given in Table III. It will be noted that paralysis developed in the treated and untreated

TABLE II.  
Effect of Insulin Shock on Experimental Poliomyelitis

Monkey * No	Dose of insulin Iv	Type of treatment	Complete paralysis in
4	3.8 units	before infection	8 days
5	"	" "	8 "
6	"	" "	12 "
7	"	" and after infection	6 "
8	"	" " " "	7 "
9	"	" " " "	7 "
10	"	" " " "	8 "
11	"	" " " "	9 "
12	"	" " " "	9 "
13	"	after infection	6 "
14	"	" "	6 "
15	"	" "	6 "
16	"	" "	9 "
17	—	—	8 "
18	—	—	7 "
19	—	—	7 "

\* All monkeys were infected intracerebrally with 0.01 cc of a 5% suspension of virus.



other monkey of approximately the same weight was subjected to similar treatment except that the drug was administered first on the day of infection and maintained thereafter. The amount of luminal given to both animals sufficed to induce profound stupor which lasted for several hours. One control monkey, infected with the same dose of virus (0.01 cc of a 5% suspension of the RMV strain) but left without drug treatment, accompanied this experiment. The results are given in Table I. It will be seen that both experimental animals developed complete paralysis in approximately the same length of time as did the control animal. Moreover the cord lesions were identical in all 3 monkeys.

*Experiment II Effect of hypoglycemic shock* A total of 13 animals were used in this experiment and arranged in 3 groups. The first group consisted of 3 monkeys which received daily doses of from 3 to 8 units of insulin by the intravenous route, beginning 3 days before intracerebral infection with poliomyelitis virus and discontinued after infection. The second group was made up of a total of 6 monkeys all of which were injected daily with similar doses of insulin beginning 3 days before infection and maintained thereafter for 6 days. The third group contained 4 monkeys in which insulin treatment of similar dosage was begun on the day of infection and continued daily for a period of 4 days. The above experiment included 3 control monkeys, infected with the same dose of virus (0.01 cc of a 5% suspension) but left without treatment. The amount of insulin used in these tests was sufficient to lower the blood sugar to the convulsive level of about 30 mg %. However the degree of systemic response varied widely among different monkeys doses of insulin which, upon first administration, induced severe shock in some animals, failing to produce the same effect in others except as the result of cumulative injections. In some instances of extremely violent symptoms it became necessary to resort to glucose administration in order to prevent coma and death. Because of these irregularities the number of shocks and their intensity varied considerably throughout the whole series but the dosage was individualized to the extent that each monkey experienced at least 3 moderately severe

TABLE I  
Effect of Narcotic Doses of Luminal on Experimental Poliomyelitis

Monkey No	Dose of Luminal Sc., mg	Type of treatment	Dose of virus (5% suspension) cc	Result
1	100 150	before and after infection	0.01	Complete paralysis, 9 days
2	100 150	after infection	"	" " 7 "
3	—	—	—	" " 8 "

In estimating the quantity of glucuronides in the urine, Quick, Sherwin, etc., used reduction methods<sup>3</sup> and assumed that the increase in reducing substances in the urine, produced as a result of the ingestion of phenylacetic acid, was due solely to an increase in urinary glucuronides.

Realizing the inadequacy of such reduction methods, Sherwin<sup>4</sup> wrote "Unfortunately, many of the studies in glucuronic acid have been confined to a qualitative or 'quantitative' test for reducing substances in the urine and attempts to check many of these results have not proved satisfactory. For this reason, and because of the importance of glucuronic acid in processes dealing with detoxication—the ingestion of phenylacetic acid has been suggested as a liver functional test—we were interested in studying the detoxication of phenylacetic acid by glucuronic acid, using a *direct* method for the determination of glucuronides. Fortunately, this was made possible by the work of Maughan, Evelyn, and Browne,<sup>5</sup> who devised a photo-

TABLE I  
Amounts of Glucuronic Acid Eliminated

Subject	Mg glucuronic acid per 24 hr sample								
	Days								
	1	2	3	4	5*	6	7	8	9
A	496	534	341	437	965	539	414	440	423
B	604	568	627	643	1115	382	476	651	465
C	418	401	530	496	992	617	520	425	421
D	502	551	615	537	1078	369	333	378	448
E	289	471	398	377	951	405	442	486	535
F	528	545	499	480	1154	477	549	549	583
G	470	503	565	658	1067	664	369	516	423
H	623	554	708	717	996	588	574	602	

\*Ingestion of 5 g of phenylacetic acid at the beginning of the 5th day

TABLE II  
Increase in Glucuronic Acid in the Urine as a Result of Feeding Phenylacetic Acid

Subject	Avg mg glucuronic acid per 24 hr in normal urines	Increase in mg glucuronic acid on ingestion of 5 g of phenylacetic acid
A	453	512
B	540	575
C	479	513
D	467	611
E	425	526
F	526	628
G	521	546
H	624	372

<sup>3</sup> Somogyi, M, *J Biol Chem* 1926, 70, 599

<sup>4</sup> Harrow, B, and Sherwin, C P, *A Text Book of Biochemistry*, 1935, p 380  
Maughan, G B, Evelyn, K. A., and Browne, I S L, *J Biol Chem*, 1938, 126, 567

TABLE III

Effect of Metrazol Shock on Experimental Poliomyelitis      Complete paralysis in 5 days

Monkey No	Dose of metrazol Iv	Type of treatment	Dose of virus (5% suspension) Ic
20	0.3 0.6 cc	before infection	0.1 cc
21	"	after infection	1 "
22	—	—	0.1 "
23	—	—	1 "

group with no perceptible difference. Upon autopsy all monkeys showed severe lesions in the cord.

*Conclusions.* Neither the administration of narcotic doses of luminal nor the production of systemic shock by means of insulin or metrazol were capable of influencing the course of experimental poliomyelitis. Moreover, the extent and severity of the lesions in the spinal cord showed no significant difference between treated monkeys and untreated control animals. Even though essentially negative, the above results are considered important in demonstrating that propagation of the virus of poliomyelitis in the central nervous system is not affected by profound cytological and metabolic changes in the nerve tissue as were produced by the methods employed in this work.

11234

### On the Detoxication of Phenylacetic Acid by Glucuronic Acid in Humans

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*From the Department of Chemistry, City College, College of the City of New York*

Using reduction methods, Quick<sup>1</sup> working with dogs concluded that phenylacetic acid is excreted in the form of its glucuronide to the extent of 34% and a considerable portion of the remainder appeared in conjugation with glycine. Working with human subjects, and using similar methods of determination, Ambrose, Power, and Sherwin<sup>2</sup> claimed that by far the largest quantity eliminated is in combination with glutamine whereas only 5% appears to be conjugated with glucuronic acid.

<sup>1</sup> Quick, A. J., *J. Biol. Chem.*, 1928, **77**, 581.

<sup>2</sup> Ambrose, A. A., Power, F. W., and Sherwin, C. P., *J. Biol. Chem.* 1933, **101**, 669.

In estimating the quantity of glucuronides in the urine, Quick, Sherwin, etc., used reduction methods<sup>3</sup> and assumed that the increase in reducing substances in the urine, produced as a result of the ingestion of phenylacetic acid was due solely to an increase in urinary glucuronides

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<sup>3</sup> Somogyi, M., *J Biol Chem* 1926, 70, 599

<sup>4</sup> Harrow, B., and Sherwin, C. P. *1 Text Book of Biochemistry*, 1935, p 380

Maughan, G. B., Evelyn, K. A., and Browne, I. S. L. *J Biol Chem*, 1938, 126, 567

electric method for the quantitative estimation of glucuronic acid and conjugated glucuronides in human urine. They made use of the color developed by the reaction of the glucuronide with naphthoresorcinol on being heated with hydrochloric acid.

The experimental procedure was as follows. 24-hour urine samples of normal male subjects were collected for 4 days. On the fifth day, 5 g of phenylacetic acid—which is as much as can be conveniently tolerated—previously neutralized with 0.2 molar NaOH, were ingested. The urine was collected for the next 5 days. The amount of glucuronide was determined in each 24-hour sample according to the method previously referred to.<sup>5</sup> The analyses were carried out in a photoelectric colorimeter of the type described by Withrow, Shrewsbury, and Kraybill.<sup>6</sup> The results with 8 subjects are shown in Tables I and II.

**Conclusion.** Dealing first with normal (control) urines, the amount of glucuronic acid in 24-hour samples varied from 350-650 mg, with an occasional lower or higher figure. These results agree well with those obtained by Maughan, Evelyn, and Browne<sup>5</sup> and by Roe and Hall.<sup>7</sup> Next, with regard to the effect of ingesting 5 g of phenylacetic acid, we find that this results in an average increase of 535 mg, an amount which corresponds to a detoxication by glucuronic acid of 7.5% of the phenylacetic acid ingested.

## 11235 P

### Metabolic Studies in Dermatomyositis, with a Note on the Effect of Wheat Germ\*

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Patients with dermatomyositis frequently have extensive changes in the skin, muscles and bones. In addition, many patients have symptoms of Ravnaud's Disease. The muscular disability in dermatomyositis can be as marked as that occurring in advanced pro-

<sup>6</sup> Withrow, R. B., Shrewsbury, C. L., and Kraybill, H. R., *Ind. Eng. Chem. Analyt. Edition*, 1936, **8**, 214.

<sup>7</sup> Roe, J. H., and Hall, J. M., *J. Biol. Chem.* 1939, **128**, 329.

\* Aided in part by a grant from the National Foundation for Infantile Paralysis, Inc.

gressive muscular dystrophy with extreme wasting and weakness. On radiographic examination, the bones often show areas of rarefaction, and in some instances complete absorption of the distal phalanges of the fingers. Deposits of calcium in the subcutaneous tissues are a frequent finding. Despite the extensive lesions that occur in dermatomyositis, few studies of the metabolism of patients with this condition have been made.

The object of the present investigations was to obtain a clearer understanding of the changes occurring in the muscles and bones. The studies included complete balances of calcium, phosphorus and magnesium at different levels of intake. In addition, the urinary excretion of creatinine and creatine was determined daily over periods of several months while the patients were receiving various substances that were being studied for their possible therapeutic effects.

*Observations and Discussion* *Calcium, Phosphorus and Magnesium* The data on the balance of calcium and phosphorus are shown in Table I. The most significant finding was the relatively low urinary excretion of calcium in both patients. Whereas the ratio of urinary calcium to fecal calcium in normal subjects usually is of the order of 1:5, the ratio in both patients was about 1:13. The metabolism of phosphorus is of interest, although probably of less significance, in that there appeared to be a diminished ability to store phosphorus when the daily intake was around 2.0 g. The magnesium balance was normal.

*Creatinine and Creatine* The data on the metabolism of creatinine and creatine are presented briefly in the chart (Fig. 1). In both patients, the urinary excretion of creatinine was diminished, and ap-

TABLE I  
Balance of Calcium and Phosphorus at Different Levels of Intake  
There were 5 days in each period during which the diet was constant. Each period was preceded by a preliminary period of 5 days during which the diet was similar to that of the experimental period.

Patient	Period	Intake		Output				Balance	
		Ca mg	P mg	Urino		Feces		Ca mg	P mg
				Ca mg	P mg	Ca mg	P mg		
1	I	1140	1485	61	830	806	368	+282	+287
	II	102	627	30	467	214	197	-142	-37
	III	2016	1900	92	1222	1560	640	+364	+38
2	I	1016	1441	49	800	760	458	+207	+183
	II	102	627	21	454	188	178	-107	-3
	III	2007	2060	96	1205	1700	888	+211	-33

electric method for the quantitative estimation of glucuronic acid and conjugated glucuronides in human urine. They made use of the color developed by the reaction of the glucuronide with naphthoresorcinol on being heated with hydrochloric acid.

The experimental procedure was as follows. 24-hour urine samples of normal male subjects were collected for 4 days. On the fifth day, 5 g of phenylacetic acid—which is as much as can be conveniently tolerated—previously neutralized with 0.2 molar NaOH, were ingested. The urine was collected for the next 5 days. The amount of glucuronide was determined in each 24-hour sample according to the method previously referred to.<sup>5</sup> The analyses were carried out in a photoelectric colorimeter of the type described by Withrow, Shrewsbury, and Kraybill.<sup>6</sup> The results with 8 subjects are shown in Tables I and II.

**Conclusion.** Dealing first with normal (control) urines, the amount of glucuronic acid in 24-hour samples varied from 350–650 mg, with an occasional lower or higher figure. These results agree well with those obtained by Maughan, Evelyn, and Browne<sup>5</sup> and by Roe and Hall. Next, with regard to the effect of ingesting 5 g of phenylacetic acid, we find that this results in an average increase of 535 mg, an amount which corresponds to a detoxication by glucuronic acid of 7.5% of the phenylacetic acid ingested.

## 11235 P

### Metabolic Studies in Dermatomyositis, with a Note on the Effect of Wheat Germ\*

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Patients with dermatomyositis frequently have extensive changes in the skin, muscles and bones. In addition, many patients have symptoms of Raynaud's Disease. The muscular disability in dermatomyositis can be as marked as that occurring in advanced pro-

<sup>5</sup> Withrow, R. B., Shrewsbury, C. L., and Kraybill, H. R. *Ind. Eng. Chem. Analyt. Edition*, 1936, **8**, 214.

<sup>7</sup> Roe, J. H., and Hall, J. M., *J. Biol. Chem.* 1939, **128**, 329.

\* Aided in part by a grant from the National Foundation for Infantile Paralysis, Inc.

slower rise in the creatinine excretion. Concomitant with these metabolic changes the clinical condition of the patients improved steadily. Muscular function increased, the tightness of the skin diminished, and the patients were able to perform muscular tasks that had been impossible for a long time.

In one series of experiments a year ago the wheat germ was incubated with normal gastric juice at 37°C for about one hour before it was administered. Patient 1 showed a definite response to wheat germ prepared in this manner. However, untreated wheat germ appeared to be equally effective when given in large amounts. When the administration of wheat germ to Patient 2 was discontinued the creatinuria increased to the level observed before the period when wheat germ was given.

The parenteral administration of riboflavin and vitamin B<sub>12</sub> daily for 6 weeks was without effect.

Patients with dermatomyositis often improve spontaneously. However, the results of this study are suggestive and warrant further investigations along these lines. Since dermatomyositis is a comparatively rare condition it is hoped that other workers will study these effects of wheat germ.

*Summary* Two patients with dermatomyositis showed a decreased excretion of urinary calcium and an apparent impaired ability to store phosphorus. The amounts of urinary creatinine were diminished proportionally to the amounts of muscular wasting. Both patients had considerable creatinuria and an impaired creatine tolerance. Prolonged administration of large amounts of wheat germ was followed by decrease in creatinuria, increase in urinary creatinine and definite clinical improvement.



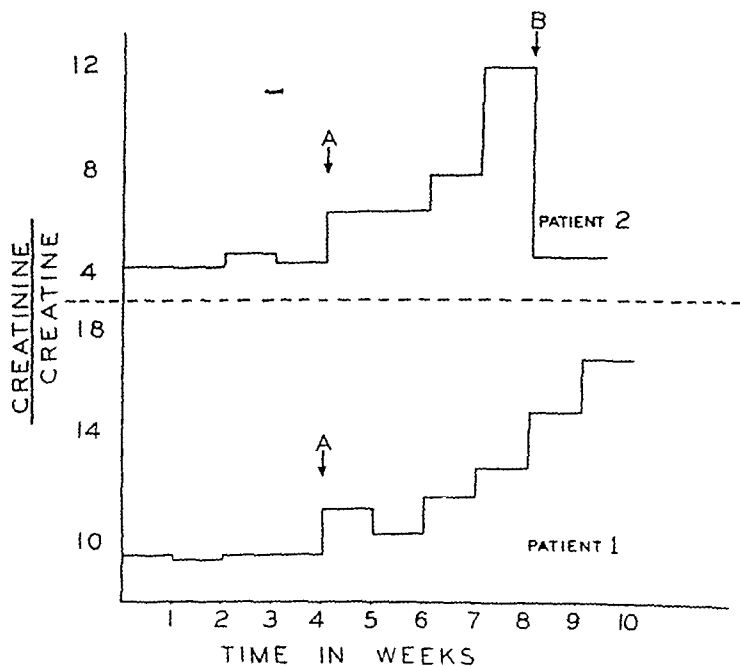


Fig. 1

Effect of wheat germ on the ratio  $\frac{\text{urinary creatinine}}{\text{creatinine}}$  Administration of 127 g wheat germ daily started at "A," and stopped at "B"

peared to be proportional to the amount of muscular wasting. In Patient 1, both the amount of muscular wasting and the decrease in urinary creatinine were of the same order as those seen in patients in the advanced stages of progressive muscular dystrophy. In this patient the amount of daily creatinine was only 0.300 g and the creatinine index was only 7.

Both patients excreted considerable amounts of creatine and showed an impaired ability to retain ingested creatine. Patient 1, with the more extensive muscular changes, excreted the larger amounts, namely around 0.350 g daily, and of an ingested dose of creatine only 30% was retained.

*Effect of Wheat Germ* Wheat germ was found to have a significant effect on the metabolism of creatine and creatinine in both patients (chart). The patients each were given 125 g of wheat germ mixed in tomato juice daily. After a period of about 2 weeks, there was a striking and progressive decrease in the creatinuria with a

EFFECT OF DRIED BILE ON GASTRIC  
HUNGER CONTRACTIONS OF DOG  
RESTING PHASE.  
DOG FASTED 25 HRS  
0.65 GM. BILE IN 10 CC. WATER.

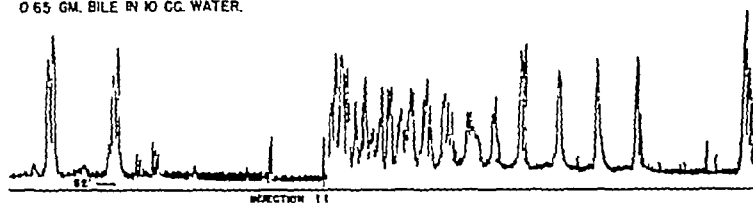


FIG 1

Note immediate effect increase in tone and production of forceful contractions after injecting whole bile into stomach during quiescent phase

EFFECT OF Na-GLYCOHYODESOXYCHOLATE ON  
GASTRIC HUNGER CONTRACTIONS OF DOG  
RESTING PHASE  
DOG FASTED 19 HRS  
0.326 GM Na GLYCOHYODESOXYCHOLATE IN 10 CC. WATER

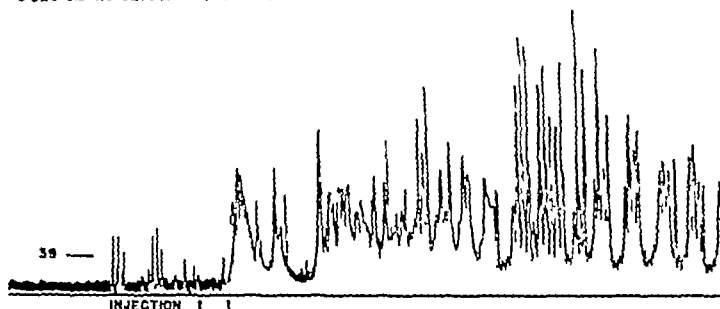


FIG 2

Note immediate effect, increase in tone and production of forceful contractions after injecting sodium  $\alpha$  glycohyodesoxycholate into stomach during quiescent phase Effect the same as obtained with whole bile

chohyodesoxycholate dissolved in water are placed in a dog's stomach during the quiescent phase gastric hunger contractions are produced (Figs 1 2) This response occurred in all of 24 experiments using whole bile and in all of 5 experiments using the bile salt

On the contrary when dried whole swine bile was placed in a dog's stomach during the contraction phase it apparently caused a definite but relatively short inhibition of contractions in 21 of 34 experiments

The same inhibitory effect on gastric contractions was noted in 6 of 13 experiments when sodium  $\alpha$ -glycohyodesoxycholate was used The effect however was less marked than that obtained with whole bile

Introduction of plain water to the amount of 10-15 cc caused mild inhibition of gastric hunger contractions in 8 of 26 times attempted

## Effect of Whole Bile and Bile Salt of Swine on Gastric Motility of the Dog

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While carrying on a clinical evaluation of the effects of feeding dried whole bile to patients suffering from a variety of lesions it was noted that the symptom of anorexia was often relieved<sup>1</sup>. Sensations of hunger occurred within a period of a few minutes to a few hours although in an occasional instance anorexia has been increased. Because of these observations we conceived the possibility that gastric contractions might be affected, or even called forth from a quiescent stomach. Accordingly we determined to investigate the response to dried bile of the stomach of fasting dogs.

**Method** Three dogs were prepared by operation with gastric fistulae after the method of Carlson<sup>2</sup> and after training and conditioning the animals, the gastric tonus and motility was measured by the balloon-manometer method. A condom balloon 8-10 cm in length was attached to the end of a No. 10 catheter and a No. 8 catheter attached alongside for gastric injection. The catheters and balloon were inserted through the gastrostomy opening into the fundus of the dog's stomach and then 50 cc of air was injected into the balloon and the catheter connected to a bromoform manometer. The 50 cc of air usually created a pressure of 3-6 cm.

The gastric tonus and motility were then recorded by Patterson's kymographic ink recording method<sup>3</sup>.

The dried whole bile used in the experiments was swine gallbladder bile prepared by vacuum distillation at low temperature\*. As sodium  $\alpha$ -glycohyodesoxycholate is the principal bile salt in swine bile<sup>4</sup> it was decided to test the effect of this salt as well as whole bile.

From analysis of the results of the foregoing preliminary study it would seem that when either dried whole swine bile or sodium  $\alpha$ -gly-

<sup>1</sup> Winfield, James M., *J. Mich. St. Med. Soc.*, 1938, **37**, 798.

<sup>2</sup> Carlson, A. J., *Am. J. Physiol.*, 1913, **32**, 369.

<sup>3</sup> Patterson, T. L., *Kongressbericht II des XVI Internationalen Physiologen Kongresses*, S. 56, August, 1938, Zurich (Schweiz).

\* Supplied by Parke Davis and Company.

<sup>4</sup> Irvin, J. Logan, Merker, Harvey, Anderson, Carl F. and Johnston, Charles G., *J. Biol. Chem.*, 1939, **131**, 439.

TABLE I.  
Distribution of Poliomyelitis Virus in Human Tissues †

Patient No	Tissues tested	Neurological signs in monkeys			Pathology in monkeys	
		First passage	Second passage			
A 2130	Spleen	Parasit. arm and leg—5 days	2 unsuccessful attempts	*	Small abscess at inoculation site	No
A 2131	Liver	Negative	"	*	microscopic lesion in spinal cord	
A 2136	Liver	"	"	*		
A 2136	Spleen	Flaccid paralysis post. extremity 5 days	1 unsuccessful attempt	*	Small abscess at inoculation site	No
A 2139	Liver	Negative		*	microscopic lesion in spinal cord	
A 2139	Spleen	Brain abscess		*		
A 2141	Liver	Negative		*		
A 2141	Mesenteric nodes	"		*		
A 2141	Spleen	"		*		
A 2141	Liver	"		*		
A 2141	Mesenteric nodes	"		*		
A 2162	Spleen	"		*		
A 2162	Liver	"		*		
A 2162	Mesenteric nodes	"		*		

\* Second passage not attempted

† No pathological examination made

‡ We are indebted for autopsy tissues to the Pathology Department of Herman Kiefer Hospital, Detroit, Mich., Joseph A. Kasper, Director

This effect is much the same as that obtained with the bile and sodium  $\alpha$ -glycohyodesoxycholate solutions although the bile inhibition occurred somewhat more consistently

If the stomach happens to be in a quiescent phase, the production of contractions might conceivably explain the prompt hunger sensations and relief of anorexia seen clinically

The mechanism is not clearly understood as yet Whether the response occurs because of an irritative phenomenon on the mucosa of the stomach or because of an effect on the intrinsic nerves cannot be stated at present

### 11237 P

## Attempts to Demonstrate Poliomyelitis Virus in Extraneural Tissues \*

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The recovery of the virus of poliomyelitis from nasopharyngeal washings<sup>1</sup> and the recent positive results with sewage,<sup>2</sup> stools of poliomyelitis patients<sup>1 3 4</sup> and healthy carriers<sup>5 6</sup> has again raised the question of virus distribution in tissues other than those of the CNS Studies with experimental animals have demonstrated the presence of viruses such as rabies<sup>7</sup> and poliomyelitis<sup>8-12</sup> in the extraneural tissues following intracerebral or intravenous injections In the present work an attempt was made to recover the virus of polio-

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\* This work was aided by a grant from the Clara Ward Seabury Clinic for Infantile Paralysis

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# Susceptibility of the Eastern Cotton Rat (*Sigmodon hispidus littoralis*) to Diphtheric Toxin

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The high degree of natural resistance of the albino rat (*Mus norvegicus albinus*) to diphtheric infection and intoxication is a matter of common knowledge. While there may be some uncertainty as to the precise amount which rats will tolerate, all investigators agree that many times the lethal dose for guinea pigs of toxin or of virulent bacilli can be injected into rats without causing death.<sup>1</sup> Armstrong<sup>2</sup> reported that a variety of Eastern cotton rats (*Sigmodon hispidus hispidus*) are apparently susceptible to infection with poliomyelitic virus. There are no data on the reaction of the cotton rat to diphtheric infection or intoxication. In view of a number of previous observations, all of which suggest some relationship between susceptibility to diphtheria and to poliomyelitis,<sup>3</sup> our interest was aroused to determine whether these rodents are as refractory to experimental diphtheria as is the albino rat.

The cotton rats were obtained in several shipments from a dealer in Florida who had trapped the wild animals in the field. They appeared to be a variety of Eastern cotton rats, listed as *Sigmodon hispidus littoralis*, which is closely related to *Sigmodon hispidus hispidus*. They were sturdy specimens, weighing from approximately 70 to 120 g and thrived when placed on a diet consisting of oatmeal, peanuts, green vegetables and apples. However upon necropsying animals that had died as the result of experimentation, it was found that they were, as a rule, heavily infected with intestinal and pulmonary worms and occasionally carried trypanosomes in their blood. The latter observation will make the subject of another

<sup>1</sup> Diphtheria Monograph, Medical Research Council, London, 1923

<sup>2</sup> Armstrong, C, *Publ Health Rep*, 1939, **34**, 1719

<sup>3</sup> (a) Zingher, A, *Am J Dis Childr*, 1917, **13**, 247, (b) Jungblut, C W, *J Immunol*, 1934, **27**, 17, (c) Foley, A R, *Canad Publ Health T*, 1934, **25**, 260, (d) Jungblut, C W, *Am J Med Sci*, 1936, **192**, 661, (e) Fischer, A L, and Stillerman, M, *Am J Dis Childr*, 1937, **51**, 984, (f) Dwyer, J M, *Med J Australia*, 1938, January 8, p 52, (g) Heaslip, W G, *Austral J Exp Biol and Med Sci*, 1938, **16**, 285, (h) Burnet, F M, Freeman M Jackson, A V, and Tush, D, *Med J Australia*, 1939, August 5, p 198

myelitis from the tissues of a monkey injected intravenously 5 days earlier with the Ayrcock strain. The animal, which showed no neurological signs, was exsanguinated and perfused with 1 liter of 0.85% saline solution. Lung, liver, spleen, intestine, brain and spinal cord were removed, ground separately, and diluted with saline. Anesthetic ether was added to a final concentration of 20%, and the suspensions were stored overnight at 5°C. The supernatant fluids were decanted, reduced *in vacuo* to one-third their original volume, and 1.5 cc of each extract inoculated intracranially into *Macacus rhesus* monkeys. The liver and spleen extracts were pooled previous to injection. The animals which received this material and the extract of lung developed quadriplegia in 9 and 24 days respectively. The other animals remained normal. The histopathology in both cases was that of acute poliomyelitis. Second transfers were successful.

Before this work could be evaluated, an unusual amount of tissue became available from human autopsies<sup>†</sup>. This material was subjected to similar treatment and injected intracranially into monkeys (Table I). The monkeys receiving spleen extracts A-2130 and A-2136 developed neurological signs, but the histopathology and monkey transfers were negative. Small pyogenic abscesses were present in both animals at the site of inoculation in the frontal region and may have caused the flaccid paralysis.<sup>11</sup>

**Conclusions.** The technic described, while similar to that used with fair results for the isolation of the virus from stools and nasopharyngeal washings,<sup>1</sup> failed to detect the agent in extraneural tissues of poliomyelitis patients. Lennette<sup>14</sup> has suggested that the virus may be present outside the CNS, but unidentifiable because of an attachment with antibody. Definite conclusions regarding the presence or absence of virus in extraneural tissues must await the development of more precise methods.

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<sup>†</sup> From the Detroit epidemic, July through October, 1939.

<sup>12</sup> Aring, C. D., *Arch Neurol and Psych*, 1940, **43**, 302.

<sup>14</sup> Lennette, E. H., *J Exp Med*, 1937, **60**, 549.

detailed report by Dr Culbertson from this laboratory. We are mentioning these facts briefly at this time since they may possibly have a bearing on the resistance of this animal to experimentally induced infections and intoxications. So far no laboratory-bred cotton rats, free from intercurrent disease, have been available.

*Diphtheric infection.* A strain of *C. diphtheriae* of known high virulence was used for this work (London *gravis* I). We are indebted to Dr Martin Frobisher, Jr. of Johns Hopkins University for kindly sending us this strain. Cultures grown for 24 hours on Loeffler slants in 4" x 1/2" tubes were suspended in saline and the bacterial emulsion in graded doses was injected subcutaneously into guinea pigs, cotton rats and albino rats. The results are given in Table I. It appears from these tests that amounts as small as 1/40 of a slant killed guinea pigs uniformly within 48 hours, whereas 1/20 of a slant sufficed to cause death in the cotton rat within 5 days. No deaths occurred in a series of albino rats injected with amounts varying from 1/10 of a slant to as much as a full slant. The lesions in guinea pigs, both local and in the adrenal, were pathognomonic of diphtheric death. A similar characteristic local lesion consisting of a gelatinous hemorrhagic exudate was but rarely obtained in the cotton rat; more often there was evidence of a localized abscess at the point of in-



FIG. 1

Local lesion in the cotton rat following subcutaneous injection of culture of *C. diphtheriae*.



TABLE I  
Infection with *C. diphtheriae* in the Guinea Pig, Cotton Rat, and Albino Rat  
(Subcutaneous injection of bacillary suspension)

<i>C. diphtheriae</i> , guinea strain	Guinea pigs No	Result		Cotton rats No	Result		Albino rats No	Result	
		Died	Lived		Died	Lived		Died	Lived
1 slant	1	1 (24h)	0	1	1 (48h)	0	1	0	1
1/2 "	1	1 "	0	1	1 (4d)	0	1	0	1
1/5 "	1	1 "	0	1	1 "	0	1	0	1
1/10 "	1	1 (48h)	0	2	2 (4d, 4d)	0	2	0	2
1/20 "	1	1 "	0	1	1 (5d)	0			
1/40 "	1	1 "	0	1	0	1			
1/100 "	1	0	1	1	0	1			
1/200 "	1	0	1	1		1			

detailed report by Dr Culbertson from this laboratory. We are mentioning these facts briefly at this time since they may possibly have a bearing on the resistance of this animal to experimentally induced infections and intoxications. So far no laboratory-bred cotton rats, free from intercurrent disease, have been available.

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FIG. 1

Local lesion in the cotton rat following subcutaneous injection of culture of *C. diphtheriae*.

jection which yielded a pure culture of diphtheria bacilli upon transfer to suitable media. This lesion is well illustrated in photograph 1. Adrenal involvement occurred irregularly and usually was not very intense. Such abscesses as have just been described were regularly seen in surviving albino rats when these animals were sacrificed during the early intervals following infection. Systematic observations on the type of cellular response of the albino rat to diphtheric infection and subsequent changes in the morphology and virulence of the bacilli will be reported elsewhere.

*Diphtheric intoxication.* The toxin employed was a sample of Park-Williams No. 8 diphtheric toxin and was obtained through the courtesy of the New York City Department of Health. At the time of running these tests the minimal fatal dose for guinea pigs was 1 cc of a 1/700 dilution. Doses of toxin ranging from 2000 mfd to 1 mfd were injected subcutaneously into guinea pigs, cotton rats and albino rats. The results are brought together in Table II. It will be seen from the figures that cotton rats succumbed uniformly to amounts of toxin as small as 5 mfd; further reduction to 2 mfd caused the death of 2 out of 4 cotton rats, while a similar number of animals survived a single fatal dose for the guinea pig. As might have been expected, albino rats tolerated inordinately larger amounts of toxin, succumbing only to 1000 and 2000 mfd. In harmony with the results obtained with bacillary infection, the fully developed picture of the local lesion, which is so characteristic for guinea pigs, was rarely seen in the cotton rat; moreover, adrenal congestion usually was not very marked and was occasionally absent. Despite prolonged observation, surviving cotton rats never exhibited any signs of postdiphtheric paralysis, excepting one animal which showed flaccid paralysis of both hind legs 6 weeks following injection with 1 mfd of toxin; this animal died 2 days later.

The above results clearly indicate the high systemic susceptibility of the cotton rat to diphtheric intoxication. The objection might be raised that some of our animals were abnormally sensitive to the toxin because a certain percentage were simultaneous carriers of intestinal parasites and of trypanosomes. Such an assumption, however, would hardly explain the closely graded correspondence throughout the entire experimental series, between the size of the inoculum—be it toxin or culture—and the response of the animal as measured by the length of its survival. In order to throw more light on this problem another experiment was carried out in which we studied the local effect of the toxin following endermal injection in

TABLE II  
Effect of Diphtheric Toxin Injected Subcutaneously into Guinea Pigs, Cotton Rats, and Albino Rats

Diphtheria Toxin, mfd	Guinea pigs, No of animals	Result		Cotton Rats No	Result		Albino Rats No	Result	
		Died	Lived		Died	Lived		Died	Lived
2000									
1000				1	1 (24h)	0	1	2 (5d, 6d)	0
700				1	1 "	0	1	2 "	0
350				1	1 (36h)	0	2	2 "	0
200				2	2 (36h, 36h)	0	1	0	0
100				2	2 (60h, 72h)	0	1	0	0
50				1	1 (72h, 72h, 72h)	0	1	0	0
25				1	1 (72h, 5d, 5d)	0	1	0	0
10				4	4 (5d, 8d, 8d, 10d)	4†	1	0	0
5				4	2 (8d, 8d)	0	1	0	0
2				4			1	0	0
1*	1	4 (48h, 48h, 72h, 96h)	0						

\*Represents 1 cc of a 1:700 dilution of toxin

†See text for development of post diphtheric paralysis and late death in one animal





FIG 2

Local lesions in the cotton rat following endermal injection of 1 and 1/2 mfd of diphtheric toxin

guinea pigs cotton rats and albino rats The results are given in Table III It will be seen that cotton rats failed to react to threshold doses of toxin such as 1/10 and 1/50 mfd which produce severe lesions on the guinea pig's skin However, comparable skin-reactions consisting of initial induration and redness followed by necrosis and ulceration were readily obtained in cotton rats by the injection of



FIG 3

Skin reaction with 1 and 1/2 mfd of diphtheric toxin in the cotton rat (Fig 2 enlarged)



FIG. 4

Skin reaction with 1/5 and 1/10 mfd of diphtheric toxin in the cotton rat

one or  $\frac{1}{2}$  mfd. These reactions are clearly shown in photographs 2 to 4. In keeping with previous experience, no skin-reactions were observed in albino rats with any of the doses of toxin included in our range.

*Conclusions* The results reported in this paper leave no doubt that the Eastern cotton rat is markedly susceptible to diphtheric infection and intoxication. While not equalling the extraordinary susceptibility of the guinea pig, the cotton rat appears to be at least many times more susceptible than the highly resistant albino rat. The significance of this observation in relation to the zoological classification of the cotton rat among the group of rodents remains to be determined.

# Mucopolysaccharide Acid of Cornea and Possible Relation to the "Spreading Factor"\*

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New York*

The nature of the mucopolysaccharide of the cornea has been much debated. A number of investigators have considered it to be a mucoitin-sulfuric acid<sup>1</sup> while others have found no sulfuric acid.<sup>2</sup> It was reported recently, on the basis of colorimetric analysis,<sup>3</sup> that the carbohydrate contained an amino sugar and sulfuric acid, but galactose instead of uronic acid. Most of the reports have been based on analyses of protein complexes ("mucoids") or of digests prepared by strong alkali.

In the past we have prepared this mucopolysaccharide acid in high yields both by alkaline digestion and by more gentle methods which avoid the use of strong alkali.<sup>4</sup> Our preparations contained one mol each of hexosamine, acetyl, uronic acid, and sulfuric acid, and had the same composition and general properties as those of the mucoitin-sulfuric acid obtained from gastric mucosa.<sup>5</sup> Good yields of glucosamine were isolated from both acids. In several important respects however, the two compounds were found to differ: (1) the acid of cornea always formed quite viscous aqueous solutions, while that of gastric mucosa did not; (2) the specific rotation of the acid from cornea was about  $-50^\circ$ , while that of the gastric mucosa was around 0 ( $+2^\circ$  to  $-8^\circ$ ) and (3) the acid from gastric mucosa was completely refractory to the specific enzyme from pneumococcus,<sup>6</sup> while the polysaccharide from cornea was hydrolyzed by this enzyme at

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<sup>1</sup>Levene, P. A., and López Suarez, J., *J. Biol. Chem.* 1918, **36**, 105; Meyer, K. and Palmer, J. W., *J. Biol. Chem.* 1936, **114**, 689.

<sup>2</sup>Mörner, C. T., *Z. Physiol. Chem.*, 1893, **18**, 213; Karlberg, O., *J. Physiol. Chem.*, 1936, **240**, 55.

<sup>3</sup>Suzuki, M., *J. Biochem. (Japan)* 1939, **30**, 185.

<sup>4</sup>Meyer, K., *Cold Spring Harbor Symposia on Quantitative Biology* 1938, **VI**, 91.

<sup>5</sup>Meyer, K., Smyth, E. M., and Palmer, J. W., *J. Biol. Chem.* 1937, **119**, 73.

<sup>6</sup>Meyer, K., Hobbs, G. L., Chaffee, F., and Dawson, M. H., *J. Exp. Med.*, 1940,



about half the rate of hyaluronic acid. From this latter finding and from the fact that the rotation of hyaluronic acid is similar to that of the cornea polysaccharide, we conclude that cornea polysaccharide is the naturally-occurring mono-sulfuric acid ester of hyaluronic acid.

This conclusion would seem to be significant in view of the recent report by Chain and Duthie<sup>7</sup> on the 'spreading factor' found in testis and bacterial filtrates. According to these workers the 'spreading factor' of testis by hydrolysis reduces the viscosity of synovial fluid and vitreous humor. These fluids were shown in our laboratory to contain hyaluronic acid as the viscous component which is hydrolyzed by a specific enzyme obtained from pneumococci, streptococci, *Cl. welchii*, and spleen. The explanation of the "spreading" action in skin suggested by the work of the English authors is that the "spreading factor" acts on a 'mucin' present as interfibrillar substance in the skin.

We were able to confirm the findings of Chain and Duthie and found further that testis extracts contain an enzyme which hydrolyzes pure hyaluronic acid as well as the polysaccharide acid of the cornea, while the mucosin sulfuric acid from gastric mucosa was found to be completely refractory. The concentration of the enzyme in testis was inferior to that of the similar enzyme found in pneumococcus and group A hemolytic streptococcus. A further distinction is the pH optimum which for the bacterial enzymes was found to be 5.8 and for the testis enzyme 4.3 with hyaluronic acid as substrate.

We believe however that the substrate in the skin on which the 'spreading factor' exerts its effect is not hyaluronic acid itself but its sulfuric acid ester. Evidence in support of this belief is furnished by the fact that such sulfuric acid esters stain meta chromatically with Toluidine blue while hyaluronic acid does not. The substantia propria of the cornea can be stained with Toluidine blue and so also are the fibrils of the corium layer of the skin. (For a beautiful illustration of the meta-chromatic staining of the cornea see ). Furthermore the protein complexes of such sulfuric acid esters are very much more stable than those of hyaluronic acid. The latter are easily brought into solution in neutral medium by dilute salt solutions, the former require concentrated salt solutions, a more alkaline reaction and often as in the case of cornea a peptizing agent such as concentrated urea. The stability of these protein complexes is apparently

<sup>7</sup> Chain, E., and Duthie, F. S. *Nature* 1939 144: 977.

<sup>8</sup> Jorpes, E., Holmgren, H., and Wihlander, O. / *Mikroskop. Anatom. Forsch.* 1937 42, 279.

responsible for the failure of some authors to obtain the cornea polysaccharide acid. By extraction with dilute salt solution, no hyaluronic acid was extracted from cattle skin. Finally, a sulfuric acid containing polysaccharide has been obtained from skin as a protein compound giving viscous solutions by Van Lier.<sup>9</sup>

Since the sclera is similar chemically and histologically to the substantia propria of the cornea, except for the absence of this polysaccharide, it becomes a problem to determine whether this polysaccharide is concerned with corneal transparency.

11240

### Can Strophanthin Maintain Adrenalectomized Mice?\*

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In a recent report by Zwemer and Lowenstein it was suggested that adrenalectomized cats can be maintained in good condition by the administration of strophanthin.<sup>1</sup> This substance was administered daily in the concentration of 15  $\mu$ g per kg of body weight.

We have attempted to extend these findings to the mouse. For these experiments 21-day-old mice were bilaterally adrenalectomized. A total of 140 operated animals were employed, 40 serving as uninjected controls while the remaining 100 animals received various concentrations of strophanthin. The strophanthin was dissolved in olive oil so that the daily dose was contained in 0.1 cc of oil. Injections were made subcutaneously, beginning 24 hours after the operation and continued for 8 days.

Table I summarizes the results of the administration of strophanthin in daily concentrations of 0.02, 0.2, 2.0, 5.0, 10.0, 20.0, 50.0, and 100.0  $\mu$ g respectively. Of 40 uninjected control animals, only 4 were alive on the 10th day after adrenalectomy. The average survival of the remaining 36 animals was 4.7 days. The administration of

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<sup>9</sup> Van Lier, E. H. B., *Z. Physiol. Chem.*, 1909, **61**, 177.

\* Supported in part by the Rockefeller Foundation and the Fluid Research Fund of the Yale University School of Medicine.

<sup>1</sup> Zwemer, R. L., and Lowenstein, B. E., *Science*, 1940, **91**, 75.

TABLE I

Survival of Adrenalectomized Mice Injected Subcutaneously with Strophanthin

Amount per day, $\mu$ g	No of animals	No of animals alive 10th day after adrenalectomy	Avg survival, days
0	40	4	4.7 (18)
0.02	15	1	3.6 (3.6)
0.2	15	1	3.8 (2.5)
2.0	15	1	4.3 (3.9)
5.0	14	1	4.2 (2.6)
10.0	14	1	4.7 (2.9)
20.0	13	0	4.5 (2.5)
50.0	7	0	2*
100.0	7	0	2*

\* All the animals in these groups were dead on the 2nd day after adrenalectomy

desoxycorticosterone acetate† in a concentration of 0.2 mg per day protected 15 of a group of 17 adrenalectomized mice

Strophanthin proved to be ineffective in the protection of the adrenalectomized mice in any of the 8 concentrations tested. No significant difference could be noted between the strophanthin injected and uninjected animals on either of the two criteria used, that is number of animals alive 10 days post-operative or the average survival of those animals dying before the tenth day. Strophanthin proved to be toxic in doses of 50 and 100  $\mu$ g, respectively. All the animals were dead within 24 hours after the first injection of strophanthin at these concentrations.

† I am indebted to Ciba Pharmaceutical Products, Inc., for the gift of desoxycorticosterone acetate

# Chemotherapeutic Evaluation of N<sup>4</sup>-n-Acylsulfanilylhydroxamides

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The antienzymatic theory of the mode of action of sulfanilamide and related compounds, as specifically represented by their inactivation of catalase is finding increasing experimental support <sup>1-10</sup> Such action, however is chiefly predicated on the formation of intermediate products of oxidation of the amino group as represented by p-hydroxylaminophenylsulfonamide The present study deals with a new series of sulfanilamide derivatives in which the amide group has been oxidized and the amino group blocked by acylation In view of the fact that such blockage has destroyed *in vitro* anticatalase activity<sup>5</sup> and to a considerable extent the therapeutic activity of previously examined derivatives it follows that the anticatalase activities of N<sup>4</sup>-n-valeryl- N<sup>4</sup>-n-caproyl- and N<sup>4</sup>-n-heptanoylsulfanilylhydroxamide\* <sup>11</sup> depends upon their oxidized amide groups

Bearing in mind the fact that the hydroxamide group is already present and does not have to be formed by the bacteria these compounds should possess powerful anticatalase and bacteriostatic properties and because of these properties superior therapeutic activity unless changed radically and quickly by the host A study of the first possibility will be reported by Main Shinn and Mellon <sup>12</sup> the study of the second is reported in the present paper

<sup>1</sup> Locke, A, Main E R, and Mellon R R *J Immunol* 1938 **30** 183

<sup>2</sup> Locke, A, Main E R, and Mellon R R *Science* 1938, **88** 620

<sup>3</sup> Main, E R, Shinn L E, and Mellon, R R *Proc Soc Exp Biol and Med*, 1938, **30**, 272

<sup>4</sup> Shinn, L E Main, E R and Mellon, R R, *Ibid* 1938 **30** 591

<sup>5</sup> Shinn, L E Main E R, and Mellon R R *Ibid* 1938 **30** 640

<sup>6</sup> Mellon, R R *Modern Hospital* 1938 October

<sup>7</sup> Locke, A., and Mellon, R R, *Science* 1939, **90** 231

<sup>8</sup> Main E R Shinn L E and Mellon, R R *Proc Soc Exp Biol and Med*, 1939, **42**, 115

<sup>9</sup> Mellon R R Locke A and Shinn L E *Am Assn for the Advancement of Science*, 1939 **11** 9S Publication No 11

<sup>10</sup> Shinn, L E Main E R and Mellon, R R *Proc Soc Exp Biol and Med*, 1939 **42**, 736

<sup>11</sup> Miller, C C Miller E and Moore M J *J Am Chem Soc* in press

\* Synthesized and donated to us by Sharp and Dohme Glenolden Penn

<sup>12</sup> Main E R Shinn I E, and Mellon R R *Proc Soc Exp Biol and Med*, 1940, **43**, 593

TABLE I  
Streptococci (Strain O 203) Infection of Mice (1,000 Fatal Doses)

Treatment	No of mice	No of deaths daily during 21 days										No of Survivors	% Survivors
		1	2	3	4	5	6	7	8	9	21		
None	75	63	7	2	2						1	None	None
Sulfanilamide	62	3	1	1	1						10	46	74
Sulfapyridine	62	1									11	50	81
N <sup>4</sup> n Valerylulfanilylhydroxamide	76	1	1		2	1		1			11	59	73
N <sup>4</sup> n Caproylsulfanilylhydroxamide	79	4	2					3	3		7	60	76
N <sup>4</sup> n Heptanoylsulfanilylhydroxamide	78	7	1	1		1	3		1	6		58	74

Infection 0.5 cc of a 10-4 broth dilution of an 18 hour broth culture intraperitoneally (1,000 fatal doses)

Treatment 10 mg of drug freshly suspended in 0.2 cc of 15% gum acacia orally 3 hours after infection, then once daily for 3 successive days (total 40 mg)

TABLE II  
Streptococci (Strain Richards) Infection of Mice (1,000 Fatal Doses)

Treatment	No of mice	No of deaths daily during 21 days										No of Survivors	% Survivors
		1	2	3	4	5	6	7	8	9	21		
None	20	14	5	1								None	None
Sulfanilamide	20	1			3	2	1	1			3	9	45
Sulfapyridine	20	1			1	1	2	1				14	70
N <sup>4</sup> n Valerylulfanilylhydroxamide	20						1	2	1	4		9	45
N <sup>4</sup> n Caproylsulfanilylhydroxamide	39		1		2	4	3	6	4	6		16	41
N <sup>4</sup> n Heptanoylsulfanilylhydroxamide	20				2	3	1	1	1	1		11	55

Infection 0.5 cc of a 10-6 broth dilution of an 18 hour broth culture intraperitoneally (1,000 fatal doses)

Treatment 10 mg of drug freshly suspended in 0.2 cc of 15% gum acacia orally 3, 22 and 46 hours after infection (total 30 mg)

TABLE III  
Type II Pneumoniae (Strept. Mitis) Infection of Mice (100 1,000 Fatal Doses)

Treatment	No. of mice	No. of deaths daily during 21 days										No. of Survivors	%
		1	2	3	4	5	6	7	8	9	10		
None	01	0	26	7	1							None	None
Sulfanilamide	53			1	3	1	7	0	1	3		6	17
Sulfapyridine	34		1	1		0	7	1				16	17
N'-N-Valeryl-sulfanilylhydroxamide	01		2	2	1	10	2	1	1			1	10
N'-N-Cephradyl-sulfanilylhydroxamide	01		1	5	1	8	1			1		1	8
N'-N-Heptanoyl-sulfanilylhydroxamide	40		1	15	14	1	3					None	None

Infection 0.5 cc of a 10 a broth dilution of an 14 hour broth culture of type II subcutaneously (100 1,000 fatal doses)

Treatment: 20 mg of drug freshly suspended in 0.2 cc of 15% gum acacia orally 4 hours after infection, then once daily for 5 consecutive days (total 120 mg)

TABLE I  
Streptococci (Strain O 203) Infection of Mice (1,000 Fatal Doses)

Treatment	No of mice	No of deaths daily during 21 days										No of Survivors	% Survivors
		1	2	3	4	5	6	7	8	9	21		
None	75	63	7	2	2						1	None	None
Sulfanilamide	62	3	1	1		1					10	46	74
Sulfapyridine	62	1									11	50	81
N <sup>4</sup> n Valeryl-sulfanylylhydroxamide	76	1	1		2	1		1		11		59	73
N <sup>4</sup> n Caproyl-sulfanylylhydroxamide	79	4	2					3	3	7		60	76
N <sup>4</sup> n Heptanoyl-sulfanylylhydroxamide	78	7	1	1		1	3	1	6			58	74

Infection 0.5 cc of a 10-4 broth dilution of an 18 hour broth culture intraperitoneally (1,000 fatal doses)  
 Treatment 10 mg of drug freshly suspended in 0.2 cc of 15% gum acacia orally 3 hours after infection, then once daily for 3 successive days (total 40 mg)

TABLE II  
Streptococci (Strain Richards) Infection of Mice (1,000 Fatal Doses)

Treatment	No of mice	No of deaths daily during 21 days										No of Survivors	% Survivors
		1	2	3	4	5	6	7	8	9	21		
None	20	14	5	1								None	None
Sulfanilamide	20		1		3	2	1	1			3	9	45
Sulfapyridine	20	1			1	1	2	1				14	70
N <sup>4</sup> n Valeryl-sulfanylylhydroxamide	20		1		2	1	2	1	4			9	45
N <sup>4</sup> n Caproyl-sulfanylylhydroxamide	39				4	3	6	4	6			16	41
N <sup>4</sup> n Heptanoyl-sulfanylylhydroxamide	20				2	3	1	1	1			11	55

Infection 0.5 cc of a 10-6 broth dilution of an 18 hour broth culture intraperitoneally (1,000 fatal doses)  
 Treatment 10 mg of drug freshly suspended in 0.2 cc of 15% gum acacia orally 3, 2, 2 and 46 hours after infection (total 30 mg)

## 11242 P

## Effect of Sulfhydryl and Other Reducing Compounds on Decarboxylation of Pyruvic Acid by Alkaline Washed Yeast \*

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Since Grassman<sup>1</sup> first showed that papain and kathepsins can be activated by cysteine or by reduced glutathione many papers have appeared in the literature dealing with the influence of sulfhydryl compounds on the activity of hydrolyzing and other enzyme systems. A search of literature has not revealed any researches concerned with the effect of these compounds on carboxylase. This paper is a preliminary report on the effect of sulfhydryl and other reducing compounds on the activity of carboxylase.

The procedure used was a modification of the method of Ochoa and Peters.<sup>2</sup> As a source of carboxylase commercial baker's yeast which had been air-dried with the aid of an electric fan for 3-4 hours or desiccated over concentrated  $H_2SO_4$  for several days was employed. It was freed from cocarboxylase by rapid washing of the yeast 2-3 times with M/10  $Na_2HPO_4$  (25-30 cc for 0.5 g yeast), and once with distilled water. The washed yeast was suspended in 5 cc M/10 phosphate buffer pH 6.2. Formation of  $CO_2$  from pyruvic acid was measured manometrically at 28° in air with conventional Warburg manometers attached to 15 cc vessels. Each vessel contained 0.5 cc washed yeast suspension, 0.1 mg Mg as  $MgSO_4$  in 0.1 cc of solution, 0.2 cc sodium pyruvate solution adjusted to pH 6.2 and containing 5 mg pyruvic acid. In each case reagents to be tested or phosphate buffer pH 6.2 were added to bring the total volume of fluid to 2 cc. The pyruvate solution was tipped into the vessel after 8-12 minutes aeration. Merck's synthetic cocarboxylase and crystalline vitamin B<sub>1</sub> were used throughout the experiments.<sup>†</sup>

The results of 5 experiments dealing with the activation of alkaline washed yeast by cysteine hydrochloride, reduced glutathione, sodium bisulfite and phenylhydrazine hydrochloride are listed in Table I.

\* This investigation has been supported by the Christine Breon Fund for Medical Research and a donation from the California Fruit Growers Exchange.

<sup>1</sup> Grassman, W., Dvellerhoff, H., and v. Schoenebeck, O., *Z. f. Physiol. Chem.*, 1929, **180**, 183.

<sup>2</sup> Ochoa, S., and Peters, R. A. *Biochem. J.* 1938, **32**, 1501.

<sup>†</sup> Our thanks are due to Merck and Company for a supply of synthetic cocarboxylase and to Squibb and Company for a supply of crystalline thiamine.



Mice infected with 2 strains of hemolytic streptococci and one strain of Type II pneumococci were treated 3 hours after infection as indicated in Tables I, II, and III. Any mouse which failed to show the infecting organism in the blood or peritoneal cavity at death was excluded from the experiment. The ratio of infection to treatment was purposely adjusted so that one-half to three-quarters of the sulfanilamide- or sulfapyridine-treated mice died. In this way better comparative values are obtained than with optimum treatment. Such values may also have greater clinical significance because of the tendency of many clinicians to cut sulfonamide medication following the first signs of improvement in order to minimize objectionable side reactions.

Reference to Tables I, II and III shows that the N<sup>4</sup>-acylsulfanilylhydroxamides are approximately equal to sulfanilamide in saving mice with hemolytic streptococcal or Type II pneumococcal sepsis.

Although the fate of the sulfonhydroxamide group cannot be followed in the body at present all 3 compounds reduce hot Benedict reagent and cold ammoniacal silver nitrate readily. They also undergo deacylation to the extent that mice given 50 mg orally show approximately 10 mg % of diazotizable material calculated as sulfanilamide, in the blood 2 hours later.

*Conclusion* N<sup>4</sup>-n-valeryl-, N<sup>4</sup>-n-caproyl-, and N<sup>4</sup>-n-heptanoyl-sulfanilylhydroxamide possess approximately the same therapeutic activity as sulfanilamide against sepsis in mice produced by 2 strains of hemolytic streptococci and one strain of Type II pneumococci.

and SH-compounds (3) The compounds may react with acetaldehyde, the chief reaction product, thus removing its influence Lohman and Schuster<sup>4</sup> have shown that acetaldehyde inhibits the system and Schubert has observed compound formation between SH compounds and the former The first possibility appears to us to be the most likely

The work is being continued and will be reported in more detail at some future date On the basis of the present work, the assay of tissues and biological fluids for cocarboxylase by the method of Ochoa and Peters<sup>2</sup> or Goodhart and Sinclair<sup>5</sup> may be open to question, since most tissues are rich in SH-compounds This may offer an explanation for the results obtained by Lipschitz, *et al*,<sup>6</sup> who found that livers of polyneuritic chicks sometimes gave values for cocarboxylase which were higher than those found for normal birds

## 11243

Toxic Effect of Human Urine on Fibroblasts Growing *in vitro*

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It has long been held that urine possesses toxic properties Bouchard<sup>1</sup> demonstrated the toxicity of urine by injecting it into the ear vein of a rabbit, determined how much was needed to kill the animal, and called quantity required per kg of body weight the urotoxic coefficient This method came to be employed for determining the toxicity of the urine in various physiological and pathological conditions Diminished toxicity in certain pathological conditions, especially uremia, was ascribed to the retention of toxic substances in the blood and was held to be the cause of some or all of

<sup>1</sup> Schubert, M P *J Biol Chem*, 1936, **114**, 341

<sup>4</sup> Lohman, K, and Schuster, P, *Biochem Z*, 1937, **204**, 188

<sup>5</sup> Goodhart, R S, and Sinclair, H M, *Biochem J*, 1939, **33**, 1099

<sup>6</sup> Lipschitz, M A, Potter, V R, and Elvehjem, C A, *Biochem J*, 1938, **32**, 474

\* With the assistance of Miss E Nussbaum

Aided by a grant from the Los Angeles Fund through Dr T Gollig

<sup>1</sup> Bouchard, quoted by Volhard, *Bergmann und Staehelin's Handbuch der Inneren Medizin*, Berlin, 1931, **6**, 720

TABLE I

Effect of Sulfhydryl and Other Reducing Agents on CO<sub>2</sub> Production from Pyruvic Acid by Alkaline Washed Yeast

Exp No	System Tested	Micro liters CO <sub>2</sub> liberated
1		in 30 min
	10 $\gamma$ B <sub>1</sub>	— 11
	10 $\gamma$ B <sub>1</sub> + 0.2 $\gamma$ cocarboxylase	23
	10 $\gamma$ B <sub>1</sub> + 2 mg cysteine HCl	130
	10 $\gamma$ B <sub>1</sub> + 2 mg cysteine HCl + 0.2 $\gamma$ cocarboxylase	177
	No B <sub>1</sub> , 2 mg cysteine HCl	111
	No B <sub>1</sub> , 2 mg cysteine HCl + 0.2 $\gamma$ cocarboxylase	110
2		in 45 min
	10 $\gamma$ B <sub>1</sub>	3
	10 $\gamma$ B <sub>1</sub> + 0.2 $\gamma$ cocarboxylase	110
	10 $\gamma$ B <sub>1</sub> + 0.2 $\gamma$ cocarboxylase + 1 mg cysteine HCl	208
	10 $\gamma$ B <sub>1</sub> + 0.2 $\gamma$ cocarboxylase + 0.5 mg cysteine HCl	174
	10 $\gamma$ B <sub>1</sub> + 0.2 $\gamma$ cocarboxylase + 0.25 mg cysteine HCl	228
	10 $\gamma$ B <sub>1</sub> + 0.2 $\gamma$ cocarboxylase + 0.1 mg cysteine HCl	201
3		in 45 min
	10 $\gamma$ B <sub>1</sub>	— 26
	10 $\gamma$ B <sub>1</sub> + 0.2 $\gamma$ cocarboxylase	154
	10 $\gamma$ B <sub>1</sub> + 0.5 mg GSH	82
	10 $\gamma$ B <sub>1</sub> + 0.5 mg GSH + 0.2 $\gamma$ cocarboxylase	205
	10 $\gamma$ B <sub>1</sub> + 0.2 mg GSH	34
	10 $\gamma$ B <sub>1</sub> + 0.2 $\gamma$ cocarboxylase	230
4		in 45 min
	10 $\gamma$ B <sub>1</sub>	— 11
	10 $\gamma$ B <sub>1</sub> + 0.2 $\gamma$ cocarboxylase	76
	No B <sub>1</sub> , no Mg	1
	No B <sub>1</sub> , no Mg, 1 mg NaHSO <sub>3</sub>	44
	10 $\gamma$ B <sub>1</sub> + 1 mg NaHSO <sub>3</sub>	49
	10 $\gamma$ B <sub>1</sub> + 1 mg NaHSO <sub>3</sub> + 0.2 $\gamma$ cocarboxylase	154
5		in 45 min
	10 $\gamma$ B <sub>1</sub>	— 15
	10 $\gamma$ B <sub>1</sub> + 0.2 $\gamma$ cocarboxylase	101
	10 $\gamma$ B <sub>1</sub> + 0.8 mg phenylhydrazine HCl	3
	10 $\gamma$ B <sub>1</sub> + 0.8 mg phenylhydrazine HCl + 0.2 $\gamma$ cocarboxylase	151
	10 $\gamma$ B <sub>1</sub> + 1.6 mg phenylhydrazine HCl	23
	10 $\gamma$ B <sub>1</sub> + 1.6 mg phenylhydrazine HCl + 0.2 $\gamma$ cocarboxylase	157

From an inspection of the table it can be seen that all these substances markedly stimulate the decarboxylation of pyruvic acid. Similar effects have been obtained with H<sub>2</sub>S. These compounds were found to be effective in amounts as low as 0.01 mg for cysteine hydrochloride, 0.1 mg for glutathione, 0.02 mg for sodium bisulfite, and 0.2 mg for phenylhydrazine hydrochloride. There are 3 possible explanations for the effects observed: (1) Free SH-groups are essential for the activity of the enzyme and since all these compounds are reducing agents they may convert SS-linkages to SH-groups; (2) The compounds, particularly cysteine and reduced glutathione, may combine with the substrate to render it more active. Schubert<sup>3</sup> has been able to demonstrate compound formation between pyruvic acid



the clinical symptoms. These experiments were the general basis for the conception of auto-intoxication.

The toxicity of the urine was later clearly demonstrated by Bruecke<sup>2</sup> who produced severe uremic symptoms in dogs by anastomosing the ureter of one kidney with the common iliac vein, the other kidney remaining intact. This was repeated and confirmed by Hartwich and Hessel<sup>3</sup> and Enderlen, Zuckschwerdt and Feucht.<sup>4</sup> These experiments led to the conclusion that the toxic effect of urine is not due to constituents preformed in the blood but that the urine must contain toxins formed in the kidney.

More recently Rohdenburg and Nagy<sup>5</sup> have demonstrated the presence in normal human urine of growth-inhibiting as well as of growth-promoting substances, using the rate of division of the protozoon *Colpidium campylum* as an index.

A report is here given of the toxic effect of normal human urine on fibroblasts growing *in vitro*. The experiments were carried out in connection with an investigation into the toxicity of uremic blood and urine, which will be reported elsewhere.

Heart fragments of uniform size from 7-day-old chick embryos were used for explantation. After 3 passages in hanging drops they were transformed to flasks and cultivated according to the standard method of Carrel. Chick's plasma diluted with Tyrode in the proportion 1:2 coagulated by one drop of diluted embryonic extract constituted the solid phase of the media.

Normal human urine (sp. gravity 1025-1030) was taken from various healthy individuals and added in varying concentrations to the flasks, the diluent used was Tyrode or embryonic extract. The urine was taken sterile or sterilized by filtering through a Jena glass filter (5/3). The pH of the supernatant fluid of the experimental flasks with concentrated urine (0.5 cc urine diluted with the same amount of Tyrode) was practically the same as that of the control flasks, it varied from 7.6 to 7.9.

In order to establish that the hypertonicity of the concentrated urine does not interfere with growth the effect on growth rate of fibroblasts of a sodium-chloride solution of the same specific gravity as the urine (1026) was examined and the experiment showed that the cultures grow in this salt solution (in amounts equivalent to the urine) just as well as in the control flasks with Tyrode.

<sup>2</sup> Bruecke, E. T., Wien *Klin. Wschr.*, 1926, **38**, 1058.

<sup>3</sup> Hartwich, A., and Hessel, G., *Klin. Wschr.* 1927, **35**, 1650.

<sup>4</sup> Enderlen, Zuckschwerdt and Feucht, *Mehner Med. Wschr.* 1928, **75**, 30.

<sup>5</sup> Rohdenburg, G. L., and Nagy, S. M., *Am. J. Canc.* 1937, **29**, 66.

has been shown that the toxicity of urine for fibroblasts is not destroyed by heat. Urine heated to 60°C and 100°C causes the same degree of inhibition of growth as unheated urine. It was also shown that the toxic substance in urine is non-dialysable. The addition to cultures of 1 cc or 0.5 cc of the residue after dialysis (corresponding more or less to the same volume of urine) results in a marked inhibition of growth (Fig. 2A and B), though somewhat less than is seen with whole urine.

The method described here of demonstrating the toxicity of urine by its effect on the growth rate of fibroblast colonies is thus exceedingly sensitive and enables us to measure the toxicity of urine with a great degree of exactitude. This method may therefore help in the investigation of the nature of the toxic substance present in urine as well as the toxicity of urine in various pathological conditions.

*Summary.* A method is described of demonstrating the toxicity of urine by its effect on the growth rate of cell colonies *in vitro*. Normal human urine in a dilution 1:5 completely inhibits the growth of fibroblasts; urine in a dilution of 1:50 still perceptibly inhibits their growth. The cells growing in media containing urine even in small concentrations exhibit signs of degeneration. Heat does not destroy this property of urine. The toxic substance is nondialysable.

11244

### Effect of Concomitant Administration of Estrogens and Progesterone on Vaginal Smear in Man \*

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Of the changes in the vaginal secretion during the menstrual cycle<sup>1</sup> those occurring in the first half concurrent with the growth and ripening of the ovarian follicle are most clearly defined and best understood. The demonstration in this laboratory<sup>2</sup> that they are similar to the smear changes induced in menopause and amenorrhea

\* Aided by grants from the Council on Pharmacy and Chemistry of the American Medical Association and the Josiah Macy, Jr., Foundation. Grateful acknowledgment is made to Dr. Erwin Schwenk of the Schering Corporation for the estradiol benzoate (Progynon B), estradiol dipropionate (Progynon DP), progesterone (Proluton) and pregnenolone (Pranone) used in these experiments, and to E. R. Squibb and Sons for their supply of estrone (Amniotin).



mone) caused a sloughing of the vaginal epithelium previously built up by estrone despite continued estrone administration

*Methods* The subjects were 9 women, 3 in spontaneous menopause and 6 with a menopausal syndrome following removal of the uterus, the ovaries, or both uterus and ovaries. The same general procedure was followed as in a previous study on the peripheral neutralization of estrogens by androgens.<sup>7</sup> A follicular type of vaginal smear was induced by estradiol benzoate, estradiol dipropionate, or estrone. Once this stage was reached the same dose of estrogen was continued along with progesterone, the dose of which was varied from time to time. In 2 cases pregnenolone was given by mouth. Vaginal smears were obtained daily and vaginal biopsies at appropriate times.

*Results* As a basis for comparison with the changes induced by this combined therapy a brief, though necessarily incomplete, description is given of the most characteristic cytological changes noted by Papanicolaou<sup>1</sup> during the post-ovulatory and premenstrual phases (1) There is usually a progressive decrease in the number of cornified cells with small pyknotic nuclei and they may entirely disappear (2) The discrete arrangement of the cells at ovulation is replaced by clumps of variable density (3) The majority of the cells are of the intermediate undifferentiated squamous type, irregularly folded or with curled edges. They assume a variety of shapes including the characteristic navicular, with large, round or oval nuclei usually predominant (4) Leukocytes usually increase in number. There is a rich bacterial growth often associated with cytolysis, and the smears have a "dirty" appearance (5) There is an increase in mucus from time to time (6) Cells from the deeper layers of the vaginal epithelium are occasionally seen.

Definite changes were recognizable in the smear within a short time after the institution of combined therapy. In about one-half of the cases they were detectable in 24 hours and in the remainder within 48 hours. The following changes were quite uniformly seen.

1 A leukocytosis of variable extent was present in all of the cases. The degree of leukocytosis varied irregularly, the smear occasionally becoming quite free of them for a day or two. In Case H S leukocytes were present in small numbers in only an occasional smear.

2 The typical cornified cells with small pyknotic nuclei rapidly diminished in number and in many instances virtually disappeared. In

<sup>7</sup> Shorr, E., Papanicolaou, G. N., and Stimmel, B. F., *Proc. Soc. Exp. Biol. and Med.*, 1938, **38**, 759.



by the administration of estrogens indicates that during the first half of the cycle the vaginal epithelium and secretion are largely if not entirely under the control of the estrogenic hormones. The peak effect of the estrogens as seen in the fully expressed "follicular picture" induced after menopause can therefore, serve as an index of the extent of spontaneous follicular activity during the normal cycle, and affords a simple histological measure of ovarian function. It can also be employed as a guide for replacement therapy with estrogens<sup>2</sup> and as a measure of ovarian stimulation by gonadotropic agents.<sup>3</sup>

The changes in vaginal smear during the second half of the cycle from the time of ovulation up to the next menstruation are less clear cut and more difficult to evaluate.<sup>1</sup> The lack of uniformity possibly arises from the more complicated hormonal pattern of this phase of the cycle. The estrogenic titer falls from its mid-menstrual peak and except for a brief moderate rise about a week premenstrually gradually sinks to the low levels characteristic of the premenstruum and the menstrual phase. The progestational hormone now appears for the first time, is elaborated for a period of about 10 days and then disappears one to 3 days before the flow.<sup>4</sup> If, as is likely, the structure of the vaginal epithelium is a resultant of the interaction of both groups of hormones, any variation in the tempo or the extent of their production could be expected to vary the cytology of the vaginal secretion.

The experiments reported in this paper are part of a study designed to analyze the influence of each of these hormonal factors on the structure of the vaginal epithelium and secretion during the second half of the cycle and to ascertain whether the vaginal smear can yield specific cytological evidence of the influence of progestin. Experiments with mice<sup>5</sup> and monkeys<sup>6</sup> have shown an antagonistic action of estrogens and progesterone on the vaginal epithelium. In the mouse progesterone like testosterone prevented cornification when given simultaneously with estrone or estradiol. In the monkey progestin (a chemically impure preparation of the corpus luteum hor-

<sup>1</sup> Papanicolaou, G. N., *Am J Anat*, 1933, **32**, No. 3 Supplement, May 15.

<sup>2</sup> Papanicolaou, G. N., and Shorr, E., *Proc Soc Exp Biol and Med*, 1935, **32**, 528, *Idem*, *J Obs and Gyn*, 1936, **31**, 806.

<sup>3</sup> Shorr, E., and Papanicolaou, G. N., *Proc Soc Exp Biol and Med*, 1939, **41**, 629.

<sup>4</sup> Venning, E. H., and Brown, J. S. L., *Endocrinology*, 1937, **21**, 711.

<sup>5</sup> Robson, J. M., *J Physiol*, 1937, **90**, 15.

<sup>6</sup> Hisaw, F. L., Greep, R. O., and Ferold, H. L., *Am J Anat*, 1937, **61**, 487.

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*Methods* The subjects were 9 women, 3 in spontaneous menopause and 6 with a menopausal syndrome following removal of the uterus, the ovaries, or both uterus and ovaries. The same general procedure was followed as in a previous study on the peripheral neutralization of estrogens by androgens.<sup>7</sup> A follicular type of vaginal smear was induced by estradiol benzoate, estradiol dipropionate, or estrone. Once this stage was reached the same dose of estrogen was continued along with progesterone, the dose of which was varied from time to time. In 2 cases pregnenolone was given by mouth. Vaginal smears were obtained daily and vaginal biopsies at appropriate times.

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most of the smears a small number of large pale flat squamous cells with small pyknotic nuclei persisted. In Case H S cornified cells continued to constitute about one-fourth of the epithelial cells but most of them were folded and wrinkled.

3 Characteristic clumping took place early. The cell aggregates were usually quite dense at the end of therapy. From time to time variation in the density of the clumping was seen.

4 Superficial squamous cells of the undifferentiated type dominated the smear picture. In the earlier stages the most characteristic change was the folding of the cell. Later, curling of the edges took place and a variety of shapes and sizes of cells was seen such as the navicular, the straplike, and even the oyster-shaped cell which has been described as characteristic of pregnancy.<sup>1</sup> In the thick smears there was considerable compression of the cells with occasional aggregates such as are also seen in pregnancy. Cells with large nuclei increased in number, but there was a persistence and frequent predominance of cells of various shapes and sizes with small pyknotic nuclei.

5 From time to time increased mucinification was seen. Bacteria appeared frequently in great abundance. In association with this, there occurred cytolysis with large numbers of free nuclei, and the smears often assumed a smudgy appearance.

6 Although a few cells from the deeper layers of the vaginal epithelium were usually seen at the end of combined treatment they were never a prominent feature.

In the 2 cases treated with pregnenolone the changes were of a similar character to those seen with progesterone but were not as marked. Apparently higher doses of this preparation should have been used.

A detailed description of the changes in the vaginal epithelium under this regime will be given in a separate report. It will suffice here to describe the major alterations noted. The biopsies taken during the induced follicular phase showed the typical increase in the height of the epithelium as compared to the untreated state. The intra-epithelial condensation or cornification of Dierks<sup>8</sup> was present and the cells of the functionalis were thick walled and flattened. Following the combined administration of estrogen and progesterone there was a definite increase in the height of the epithelium contributed largely by the functionalis. The intra-epithelial zone of cornification became less prominent and in many instances disappeared. The walls of the cells of the functionalis became thinner,

<sup>8</sup> Dierks, K, *Arch f Gynack* 1927, 130, 46

TABLE I  
Tabulation of the Dosage of Estrogen and Progesterone Administered Simultaneously to Women in Menopause

Case	Condition	Estrogen		Progesterone mg (number days)
		Preparation	Estrogen unit and maintenance dose R.U. per day	
9 B	Natural Menopause	Lastradiol Benzonte	3,000	10 (4), 20 (9)
10 G	" "	Istrone	1,500	10 (6), 20 (6)
11 A	" "	" "	3,500	15 (6)
12 K	Surgical Castrate	Lastradiol Benzonte	4,000	10 (6), 20 (6), 35 (6), 100 (6) *
13 B	" "	" "	3,000	10 (6), 25 (5), 25 (9)
14 C	" "	" "	7,500	10 (7), 20 (4), 35 (5)
15 L	" "	" "	10,000	10 (6), 25 (7)
16 S	Surgical—ovary present	" Dihyponate	5 mg $\times$ wick	25 (12)
17 D	Surgical Castrate	" Benzonte	1,000	50 (7) *, 100 (9) *

\*Pregninolone orally

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<sup>1</sup> Dierks, K, *Arch f Gynak* 1927, 130, 46

## 11245 P

Possible Relation of the Inclusion Body of Trachoma  
to Pathogenesis \*

L A JULIANELLE

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Among other reasons for regarding the causative agent of trachoma as a virus is the cytoplasmic inclusion of the epithelial cells of the conjunctiva and occasionally even of the cornea. Marshalling together evidence that is essentially morphological a number of investigators beginning with Halberstaedter and Prowazek<sup>1</sup> have attempted to prove that the inclusion represents a mass or "colony" of infectious units. While conducting a broader study† on the inclusion of trachoma and clinically allied diseases in this laboratory observations have been made by indirect approach which are sufficiently interesting on this phase of the subject to merit their publication.<sup>2</sup>

In testing out the infective capacity of conjunctival tissues from different patients with trachoma monkeys in one group were inoculated with individual tissues all of which were found to contain inclusions. In a second group monkeys were inoculated in a similar manner with tissues lacking inclusions. In a third group, monkeys were inoculated with pooled tissues some of which were inclusion-bearing and others inclusion-deficient. A study of the data pertinent to these experiments reveals several interesting and suggestive results. Thus, 70 monkeys (*Macacus rhesus*) were inoculated with individual tissues from 37 patients, all containing inclusions. Typical experimental trachoma occurred in 35, or 50% of the animals tested. On the other hand, 158 monkeys were inoculated with separate tissues from 112 patients in whom inclusions were not demonstrated. Of these animals 35, or 22% were specifically infected. The pooled tissues which were derived from 89 patients 44 with and 45 without inclusions, were inoculated

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\* Conducted under a grant from the Commonwealth Fund of New York

† A complete report will be published in the *American Journal of Ophthalmology*

<sup>1</sup> Halberstaedter, L. and Prowazek, S. *Deut med Woch*, 1907, **33**, 1285

<sup>2</sup> For a review of the subject of inclusions in trachoma, see Julianelle, L. A., *The Etiology of Trachoma* Chap. VI, The Commonwealth Fund Division of Publications, New York, 1938

the cells wider, presenting in the most extreme cases a chicken-wire appearance. This picture is similar to that described by Dierks, and by Traut, Bloch and Kuder<sup>9</sup> during the proliferative phase, and in the most extreme hyperplasias, resembles the picture seen in pregnancy<sup>9, 10</sup> where the production of both groups of hormones is at a very high level.

Symptomatically, previous relief of the menopausal symptoms induced by estrogen persisted throughout the concomitant administration of progesterone. Occasionally increased fullness and tenderness of the breasts was noticed.

*Discussion* It is apparent that the concomitant administration of progesterone and estrogen causes profound alterations in the vaginal smear as compared with the picture seen with estrin alone. Virtually all of the changes seen during the second half of the cycle are reproduced. Since the amount of estrogen was constant throughout, it was not to be expected that the changes would occur in the exact sequence seen during the normal cycle. The inference seems warranted that the changes taking place during the second half of the cycle result not only from the diminution in estrin production but also from the active influence of the progestational hormone in modifying the effect of estrin on the vaginal epithelium. This conclusion must also be true for pregnancy where both groups of hormones are present in such large amounts.

No sloughing of the epithelium as observed in the monkey was seen in this group of women although the proportion of progesterone to estrin was frequently as great as that employed in the animal experiments. The human epithelium appears to be more sensitive to progesterone than that of the mouse in that much less progesterone in proportion to estrin is effective in abolishing cornification.

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<sup>9</sup> Traut, H. F., Bloch, P. W., and Kuder, A., *Surg Gynec and Obst*, 1936, **63**, 7.

<sup>10</sup> Stieve, H., *Ztschr f mikros anat Forsch*, 1925, **3**, 307.

Induction of Water Drive in *Triturus viridescens*  
With Anterior Pituitary Extract \*

C S CHADWICK (Introduced by Karl E. Mason)

From the Department of Biology, Vanderbilt University, and the Highlands  
Biological Laboratory Highlands, N. C.

In certain well established localities the American newt, *Triturus viridescens*, shows two well-defined habitats in its life cycle. The larval and adult phases live in water while the immature phase, representing some 3 to 5 years of the cycle, lives on land. In this interesting life-cycle 2 migrations occur, that from water to land following metamorphosis and that from land to water as maturity is reached. In an attempt to determine whether or not these migrations—particularly that from land to water—are brought about by the endocrine glands Reinke and Chadwick<sup>1</sup> were able to induce the land phases to assume a water habitat, long before they would do so normally, by giving them intramuscular implants of pituitaries from the adults. By appropriate tests the water drive-inducing factor was shown to be a product of the anterior lobe of the pituitary. They were able to show later that this action of the pituitary occurs in the absence of both the thyroids and gonads.<sup>2</sup>

Is the factor which induces the water drive peculiar to *Triturus* or is it a hormone distributed widely among the vertebrates but with a specific effect in *Triturus*? If generally present in the vertebrates, with what known pituitary principles is it to be identified? The nature of its effect in *Triturus* is such that one is led to believe that the same pituitary principle which is responsible for growth in the body generally also induces migration to water as maturity is reached. In order to test the presence of the water drive factor in at least one other vertebrate class and at the same time to test the growth hormone as possibly being the cause of it an attempt has been made to induce the water drive with a commercial extract of sheep pituitary which contains the growth-promoting factor (Antuitrin G). A total of 49 land phases (red eft) of *Triturus* were injected and set up for observation according to procedures previously described.<sup>2</sup>

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\* Antuitrin G, Parke Davis

<sup>1</sup> Reinke, E. E., and Chadwick, C. S., *Proc. Soc. Exp. Biol. and Med.* 1939, 40, 691.

<sup>2</sup> Reinke, E. E., and Chadwick, C. S., *J. Exp. Zool.* 1940 83 223



into 106 monkeys and of these, 41, or 38%, were infected. If the figures for monkeys infected in each group are plotted in the form of a curve, the result is practically a straight line, with inclusion-bearing tissues inducing most infections, the inclusion-lacking tissues inducing least or approximately half as many infections, and the tissues pooled as indicated occupying an almost mathematically half-way position between the two extremes.

Because the above result seemed to have a certain significance, the data were reassembled to determine whether the same conclusions were approachable on some other basis. Thus, of 18 separate experiments performed with tissues containing inclusions, 13, or 72%, were considered positive (i.e., animals were specifically infected). Of 42 experiments conducted with material lacking inclusions, 14, or 33% were positive, while of 24 experiments with mixed tissues, 14, or 58%, were positive. A curve representing the data runs a remarkably parallel course with that of the preceding observations.

This is not the place to discuss the possible significance of these results. However, the implications are obvious that the presence of inclusions accompanies greatest infective capacity of trachomatous tissues, and the absence of inclusions the least, while both tissues pooled approximately on a 1:1 basis approach a value half-way between both—a result certainly to be anticipated if the pooling were to represent a simple process of dilution. While it cannot be said that the evidence is conclusive, the data are of sufficient magnitude to suggest not an accidental outcome, but an actual relation of inclusion to infectivity, even to the extent that the inclusion represents an agglomeration of virus particles. This, however, is an opinion requiring further elaboration before it can be finally accepted.

11246

Induction of Water Drive in *Triturus viridescens*  
With Anterior Pituitary Extract \*

C S CHADWICK (Introduced by Karl E. Mason )

From the Department of Biology, Vanderbilt University and the Highlands  
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<sup>1</sup> Reinke, E. E., and Chadwick, C. S., *Proc. Soc. Exp. Biol. and Med.* 1939, 40, 691

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A preliminary test was made on 18 efts. Six, ranging from 60 to 90 mm in length and given 3 injections of 1 RU each at 48-hour intervals, all entered water within 122 hours. Six, ranging from 83 to 95 mm in length and given 3 injections of 1 RU each at 24-hour intervals, entered water within 92 hours. Six, 78 to 93 mm efts each given 4 RU in 2 equal amounts 24 hours apart, entered water in 72 to 100 hours. All 18 efts molted by the 3rd day following the initial injection and all had entered water by the 5th day. Their adaptation to water was apparently complete and by the 10th day their color had undergone a profound change from the red of the land phase to the green and yellow of the adult water stage. Controls, either uninjected or injected with tap water or Antuitrin S† did not enter water nor did they molt nor show any change in color.

In an attempt to determine the threshold dosage of the extract necessary to induce the water drive, 12 efts ranging from 50 to 90 mm in length, were each given single injections varying from 0.3 to 2 RU. Eleven of the 12 entered water at intervals of 3 to 10 days. Only 2, each receiving 2 RU doses, remained permanently adapted. The others were in and out of water rather indifferently and, when it appeared that there could be no correlation between dosage and time required to enter water to stay, each eft was given enough additional extract to make a total dosage of 3 units. All of these efts molted and, with the exception of the one 50 mm eft, were well adapted to water when killed 2 to 3 weeks after the initial injection. These observations indicate that 2 to 3 RU of Antuitrin G are necessary to induce the water drive, providing the injected animals are above 50 mm in length.

In order to check further the failure of the extract to induce small efts to go to water, 7 efts, ranging from 46 to 55 mm in length, were each given 6 half-unit injections at 12-hour intervals. Although all molted on the 2nd and 3rd days and entered water by the 6th day, none of them remained consistently in water during the 3-week period of observation. Additional injections of the extract failed to make them become completely adapted to water.

Since Antuitrin G contains both the gonadotropic and thyrotropic anterior pituitary factors, it was considered of interest to see if the extract would induce the water drive in efts deprived of their gonads and thyroids. Six gonadectomized efts ranging from 75 to 90 mm in length and given 3 RU in 3 days responded identically with the animals in the preliminary test. Only 1 of 6 thyroidectomized efts

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† Parke Davis Co. This extract contains the anterior pituitary like factor and had been shown previously not to be effective in inducing the water drive.

of similar size and given the same treatment entered water and none molted

To summarize briefly, 35 large efts ranging from 60 to 95 mm in length, either normal or gonadectomized were driven to water within 5 days by injections of 2 to 3 RU of mammalian pituitary extract. Similar quantities failed to cause 8 efts less than 55 mm in length and 5 of 6 large thyroidectomized efts to enter water.

While mammalian pituitary extract does not consistently induce the water drive in small efts nor in efts deprived of their thyroids the fact that it invariably causes large normal or gonadectomized efts to migrate to water is proof that the water-drive factor is not restricted to *Triturus* alone. Although the return of *Triturus* to water is necessary for reproduction, the gonads seem not to be concerned in any way with the initiation of the water-drive. The failure to induce the water drive consistently in thyroidectomized efts by injection of mammalian extracts when implants of adult *Triturus* pituitary readily do so<sup>2</sup> indicates either a species difference or that some qualitative change has been brought about in the mammalian factor due to the manner of extraction. The failure to induce the drive in small efts is also quite interesting but before any conclusions can be drawn as to these failures a greater series of thyroidectomized and small efts must be injected. The acceleration of the acquisition of adult characteristics along with the water drive in large efts by injection and in efts of any size by implantation of adult *Triturus* pituitary together with the fact that the water drive is induced by a recognized growth hormone-containing extract indicates that the growth hormone is also the water-drive hormone.

## The Bacteria-Free Culture of a Nematode Parasite

R W GLASER (Introduced by Carl TenBroeck)

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It has been possible for some time to grow the entire life cycle of *Neoaplectana glaseri*,<sup>1</sup> a nematode parasite of the Japanese beetle, in cultures in which bacterial and fungous growths have been inhibited in various ways but not eliminated.<sup>2</sup> These contaminants undoubtedly introduced a high degree of variability into the results obtained. It therefore seemed advisable to attempt to rear this parasite in cultures free from bacteria.

Lapage<sup>3</sup> and Glaser and Stoll<sup>4</sup> developed technics whereby the second ecdysis of strongyloid nematode larvae was easily and consistently obtained in quantity under sterile conditions. It was found necessary to modify one of these technics slightly for work with *Neoaplectana*. Cultures prepared in the routine manner were permitted to develop for 10 to 15 days, at which time the majority of the parasites were second-stage larval forms in their third or fourth generation.\* These were removed from the surface of the solid medium and washed with water until free of much debris. To remove the dead larvae they were then filtered through 2 layers of lens paper supported by fine gauze. The larvae were ensheathed by aerating them in 25 cc of water for 3 to 4 days with a change of clean water 3 times daily. They were then washed in 25 cc lots of sterile water 3 times each day for 3 days, after which they were treated for 30 minutes to 1 hour with Labarraque's solution (sodium hypochlorite) at a dilution of 1 part to 40 or 50 parts of water. The nemas were again washed 3 times in sterile water, and then placed in

<sup>1</sup> Steiner, G, *J Washington Acad Sci* 1929, **19**, 436

<sup>2</sup> Glaser, R W, *Science*, 1931, **73**, 614, Cire 211, Dept of Agric, State of New Jersey, 1932, *Studies from The Rockefeller Institute for Medical Research*, 1932, **83**, 521, McCoy, E E, and Glaser, R W, Cire 265, Dept of Agric, State of New Jersey, 1936, McCoy, E E, and Girth, H B, Cire 285, Dept. of Agric, State of New Jersey, 1938

<sup>3</sup> Lapage, G, *J Helm*, 1935, **13**, 103, *Parasitology* 1935, **27**, 186

<sup>4</sup> Glaser, R W, and Stoll, Norman R, *J Parasit*, 1940, **26**, 87

\* The ensheathed second stage larva is the only stage capable of surviving free in nature and represents the invasive form which must penetrate into a host (Japanese beetle grub) to continue its development

water about 5 mm deep for from 15 to 20 hours † The next day the nemas were again treated with Labarraque's solution and then washed 3 times, followed by another 15 to 20 hours' sojourn in a small amount of water This was followed by a third treatment with Labarraque's solution and 3 more washings with sterile water Finally, the nemas were again passed through sterile lens paper, to remove any worms that had died during the manipulations, and the viable forms were stored in shallow water until used.

When the above procedures were carefully followed sterile larvae were obtained, shown by the fact that no bacterial growth occurred when they were cultured on standard laboratory media under both aerobic and anaerobic conditions When occasional contaminants appeared later after a prolonged incubation period, such cultures were either placed aside for collateral observations or discarded

At first it was thought necessary to have a solid substrate to facilitate the movements and ecdyses of *Neoplectana* Neutral veal infusion agar slants may be used, but a simple substrate of 2% agar prepared with 0.5% sodium chloride solution answered just as well Ten cc of the melted agar were slanted in culture tubes measuring 180 x 22 mm About one gram of animal tissue, removed under sterile conditions, was then placed at the base of the slant, and 2 or 3 drops of sterile 0.5% salt solution were added Each tube was inoculated with  $\pm 200$  of the previously sterilized second-stage larvae and then sealed by pouring melted sealing wax over the cotton stopper previously trimmed and pushed down into the tube for approximately half an inch When hard the sealing wax was perforated by a hot wire Sealing in this manner prevents excessive evaporation without excluding oxygen Recently we have also sealed many tubes with 'Parafilm' perforated with a few needle pricks and have found this satisfactory The sealed tubes were held in a slanted position and incubated at room temperatures, 22-28°C

Eighteen- to 20-day-old mouse embryo, beef kidney, and rabbit ovary and kidney have all been found to support growth The last proved to be the easiest to manipulate and to give the best growth Consequently it has been used for most of the work In tubes containing approximately one gram of rabbit kidney the tissue is almost completely digested in from 18 to 24 days, at this same period of incubation growth has reached its maximum and second-stage larvae predominate Cultures may be held for at least 3 months without transplantation but to maintain vigorous growth transfers are made

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† During this time interval *Neoplectana* digested and excreted the greater part of its intestinal flora

## The Bacteria-Free Culture of a Nematode Parasite

R W GLASER (Introduced by Carl TenBroeck)

From the Department of Animal and Plant Pathology, Rockefeller Institute for Medical Research, Princeton, N J

It has been possible for some time to grow the entire life cycle of *Neoaplectana glaseri*,<sup>1</sup> a nematode parasite of the Japanese beetle, in cultures in which bacterial and fungous growths have been inhibited in various ways but not eliminated.<sup>2</sup> These contaminants undoubtedly introduced a high degree of variability into the results obtained. It therefore seemed advisable to attempt to rear this parasite in cultures free from bacteria.

Lapage<sup>3</sup> and Glaser and Stoll<sup>4</sup> developed technics whereby the second ecdysis of strongyloid nematode larvae was easily and consistently obtained in quantity under sterile conditions. It was found necessary to modify one of these technics slightly for work with *Neoaplectana*. Cultures prepared in the routine manner were permitted to develop for 10 to 15 days, at which time the majority of the parasites were second-stage larval forms in their third or fourth generation.\* These were removed from the surface of the solid medium and washed with water until free of much debris. To remove the dead larvae they were then filtered through 2 layers of lens paper supported by fine gauze. The larvae were ensheathed by aerating them in 25 cc of water for 3 to 4 days with a change of clean water 3 times daily. They were then washed in 25 cc lots of sterile water 3 times each day for 3 days, after which they were treated for 30 minutes to 1 hour with Labarraque's solution (sodium hypochlorite) at a dilution of 1 part to 40 or 50 parts of water. The nemas were again washed 3 times in sterile water and then placed in

1 Steiner, G, *J Washington Acad Sci*, 1929, **19**, 436

2 Glaser, R W, *Science*, 1931, **73**, 614. Cire 211, Dept of Agric, State of New Jersey, 1932, *Studies from The Rockefeller Institute for Medical Research*, 1932, **88**, 521, McCoy, E E, and Glaser, R W, Cire 265, Dept of Agric, State of New Jersey, 1936, McCoy, E E, and Girth, H B, Cire 285, Dept of Agric, State of New Jersey, 1938

3 Lapage, G, *J Helm*, 1935, **13**, 103, *Parasitology*, 1935, **27**, 186

4 Glaser, R W, and Stoll, Norman R, *J Parasit*, 1940, **20**, 87

\* The ensheathed second stage larva is the only stage capable of surviving free in nature and represents the invasive form which must penetrate into a host (Japanese beetle grub) to continue its development

acetate\* on the fall in plasma volume which occurs in acute intestinal obstruction in dogs."

The dose of hormone used was arbitrarily determined. One or  $1\frac{1}{2}$  mg per day is necessary to sustain an adrenalectomized dog.<sup>9</sup> Since we were dealing with a rapidly exhausting state in our experiments we injected 5 mg at the beginning of the experiment and repeated the dose once or twice at 4-hour intervals.

**Results** In Table I it will be observed that in dogs not receiving hormone the longer the distention of the small intestine continued the greater was the loss of plasma. This averaged 35% after 4-6 hours and 55% after 18-23 hours. The detailed data of these experiments already published show no great disparity between individual measurements and the average figure.

Of the 4 dogs which received desoxycorticosterone the first lost 38% of the plasma in the first 6 hours. There was however no significant change in the following 6 hours. The final measurement showed an actual return of a large part of the lost plasma to the circulating blood—an observation never observed in continuously distended dogs not receiving hormone. The first dose of hormone in this dog was given intramuscularly while all other doses in this and the other 3 dogs were given intravenously. In the second dog of this group there was an increase in the loss of plasma from 9%

TABLE I  
Distention of Small Intestine  
% Change in Plasma Volume

	After 4-6 hr	8-12 hr	18-23 hr
D	-36 (9)	-43 (2)	-55 (5)
D (Hormone)	-38 -9 - -5	-40 -21 +49 +5	-16 - +24 -
C	-8 (7)	-	-12 (7)

D—Distended dogs

D (Hormone)—Distended dogs receiving desoxycorticosterone

C—Control dogs without distention or with distention of other hollow organs

Figures in parentheses represent the number of separate observations, the results of which were averaged to obtain the accompanying figures. The data of the D (Distended) dogs has already been published<sup>7</sup> (Table I, Group 3). The individual results are sufficiently close to justify using the average result. The data of the C (Control) dogs is partly from the same published data<sup>7</sup> (Table I, Groups 1 and 2) and from data to be published in which the individual results are likewise sufficiently uniform to permit use of the average result. The hollow organs distended without marked loss of plasma were the colon and the gall bladder.

\* We are indebted to Ciba & Co. who supplied this material.

<sup>7</sup> Gendel, S., and Fine, I. *Annals Surg.* 1939, **110**: 27.

<sup>9</sup> Thorn, G. W., Engel, L. I., and Eisenberg, H. *Bull. J. H. H.*, 1939, **64**: 155.



every 3 weeks. Sterile nematodes have to date been maintained without loss through 14 transfers or approximately 50 generations. With the methods previously used it was necessary to add certain accessory growth factors in order to maintain the cultures longer than 21 to 32 generations.<sup>1</sup> The nematodes have also been cultured in Erlenmeyer flasks containing 0.5% NaCl solution to a depth of 3 to 4 mm in which are placed pieces of sterile rabbit kidney, and in liquid media containing kidney extracts devoid of particulate matter.

On agar slants containing rabbit kidney the number of nematodes found after incubation for 18 days is approximately 150,000. When grown on Petri dishes 5.5 cm in diameter, containing 2% dextrose agar and living yeast, the yield was about 40,000. Contamination of the rabbit kidney causes a marked reduction, and in some cases an absence, of growth.

In view of the fact that this parasitic nematode can be grown on both liquid and solid media free from bacteria it will be possible to study its nutritional and other requirements and to test the effects of various vitamins and hormones.

## 11248 P

## Effect of Desoxycorticosterone on Plasma Volume in Intestinal Obstruction

JACOB FINE, FELIX FUCHS AND JEROME MARK

*From the Surgical Departments of the Beth Israel Hospital and the Harvard Medical School, Boston, Mass*

Adrenal cortical hormone restores the reduced blood volume in Addison's disease<sup>1</sup> and prevents the drop in plasma volume said to occur during ether anesthesia.<sup>2,3</sup> This communication reports some experimental observations on the effect of desoxycorticosterone

<sup>1</sup> Glaser, R. W., *J. Exp. Zool.*, in press.

<sup>2</sup> Thorn, G. W., Howard R. P., and Emerson K., Jr., *J. Clin. Invest.*, 1939, **18**, 449.

<sup>3</sup> Loeb, R. F., Atchley, O. W., Ferrebee, J. W., and Ragen, C., *Trans. Assn. Am. Phys.*, 1939, **54**, 285.

<sup>4</sup> McAllister, F., and Thorn, G. W., *Proc. Soc. Exp. Biol. and Med.* 1937, **36**, 736.

<sup>5</sup> Ragen, C., Ferrebee, J. W., and Fish, G. W., *Proc. Soc. Exp. Biol. and Med.*, 1939, **42**, 712.

acetate\* on the fall in plasma volume which occurs in acute intestinal obstruction in dogs

The dose of hormone used was arbitrarily determined. One or  $1\frac{1}{2}$  mg per day is necessary to sustain an adrenalectomized dog.<sup>6</sup> Since we were dealing with a rapidly exhausting state in our experiments we injected 5 mg at the beginning of the experiment and repeated the dose once or twice at 4-hour intervals.

**Results** In Table I it will be observed that in dogs not receiving hormone the longer the distention of the small intestine continued the greater was the loss of plasma. This averaged 35% after 4-6 hours and 55% after 18-23 hours. The detailed data of these experiments already published,<sup>7</sup> show no great disparity between individual measurements and the average figure.

Of the 4 dogs which received desoxycorticosterone the first lost 38% of the plasma in the first 6 hours. There was however no significant change in the following 6 hours. The final measurement showed an actual return of a large part of the lost plasma to the circulating blood—an observation never observed in continuously distended dogs not receiving hormone. The first dose of hormone in this dog was given intramuscularly while all other doses in this and the other 3 dogs were given intravenously. In the second dog of this group there was an increase in the loss of plasma from 9%

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Gendel, S., and Fine, J., *Annals Surg.* 1939, **110**, 25.

<sup>6</sup>Thorn, G. W., Engel, L. I., and Eisenberg, H., *Bull. J. H. H.*, 1939, **64**, 155.

after the first 6 hours to 21% after 12 hours. These figures are of a much lower magnitude than was observed in the dogs not receiving hormone and to this extent the hormone may be considered to have exerted a protective influence. In the third dog an astonishing increase in plasma above normal was noted in the 8-12-hour period of distention and though not sustained in the subsequent interval before death occurred, a substantial positive effect seems to have been maintained by the hormone. The measurements of plasma loss in the fourth dog also show the capacity of the hormone to prevent a loss of plasma. The effect of desoxycorticosterone is therefore one of counterbalancing the action of the agent responsible for the loss of plasma.

The possible influence of other factors on the plasma volume changes are excluded by the results of a group of 7 control experiments which included 2 intact dogs under nembutal anesthesia, 2 dogs with obstruction but no distention, 1 dog with distention of the gall bladder, and 2 with distention of the colon. None of these animals showed any marked loss of plasma at any time. The observations in the last 3 dogs of this series, which suggest that the plasma loss from distention of a hollow viscus is more or less specific for the small intestine, will be published elsewhere in more detail.

The apparently favorable response to the trauma of distention in the 4 dogs receiving hormone applies, so far as our data permit a judgment, only to the plasma volume. There was no prolongation of life by the hormone. This may be due to the undesirable effects of overdosage.<sup>2</sup>

*Conclusion* The marked fall in plasma volume observed in dogs subjected to continuous distention of the small intestine is at least partly prevented by the intravenous administration of desoxycorticosterone.

## Cutaneo-Visceral Vasomotor Reflexes in the Cat

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St. Louis, Mo*

The use of localized cutaneous stimulation in the treatment of visceral and other deeply located lesions has been based mainly on empirical considerations. Certain investigators, particularly Boas<sup>1</sup> and Freude and Ruhmann,<sup>2</sup> advanced data which support the assumption that localized cutaneous stimulation by means of warm applications results in vasodilatation in the corresponding segments of the gastro-intestinal tract. Certain data also support the assumption that localized cooling of the skin results in vasoconstriction in the viscera in the corresponding segments. There is no general agreement regarding the vascular reactions in the viscera elicited by localized cutaneous stimulation or the mechanisms through which such reactions are brought about.

The present series of experiments has been carried out to determine more accurately than has been indicated by previous studies whether appreciable circulatory changes in visceral organs can be brought about by localized cutaneous stimulation and whether the changes which occur represent direct effects of the stimulation employed or reflex phenomena.

Decerebrate preparations of the cat have been used in order to avoid the vitiating effects of anesthesia. The stimulating agents employed have been warm and cold applications and vacuum cups applied to the skin of the back and lateral surfaces of the trunk from which the hair had been removed. The circulatory changes brought about in the stomach and intestine were observed with the viscus exposed through a midventral incision, and recorded by means of photography and plethysmograph records.

Moderate cooling of the skin of the back or lateral surface by means of cold applications from the fifth or sixth thoracic segment caudalward consistently resulted in vasoconstriction in the stomach and intestine. Moderate warming of the skin in the same areas by means of warm applications at approximately 45°C consistently resulted in vasodilatation in the stomach and intestine (Fig 1). Stimulation of the skin by means of vacuum cups resulted in

<sup>1</sup> Boas, I., *Deutsch med Wochenschr* 1926, 52, 349.

<sup>2</sup> Freude, E., and Ruhmann W., *Z f d ges exp Med* 1926 52, 333.

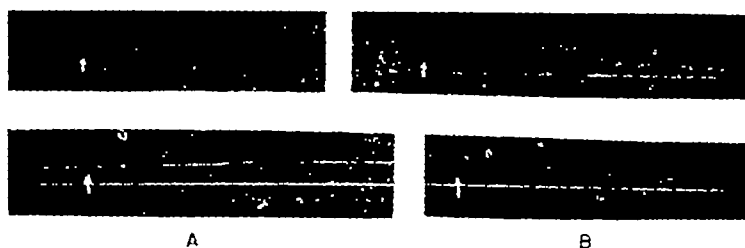


FIG 1

Plethysmograph records of a loop of the small intestine of the decerebrate cat during localized cutaneous stimulation by means of warm and cold applications. Initiation of stimulation is indicated by arrows

A, Records of responses to warm applications

B, Records of responses to cold applications

vasodilatation in the corresponding portion of the gastrointestinal tract in approximately the same degree as moderate warming of the skin in the same area

Photographs of segments of the gastrointestinal tract obtained during intervals of vasodilatation due to local warming of the skin and during intervals of local cooling of the skin, compared with those obtained in the absence of specific cutaneous stimulation indicate that the changes in calibre elicited by means of the stimulation employed are more marked in the smaller blood vessels than in the larger ones. Direct observations under low magnification also support this conclusion. The changes observed and recorded are of sufficient magnitude to warrant the conclusion that the volume of blood circulating through the affected segments of the gastrointestinal tract is markedly increased by local warming and markedly decreased by local cooling of the skin.

The circulatory changes in the gastrointestinal tract observed in these experiments cannot be explained on the assumption that the gastrointestinal blood vessels were influenced directly by the stimulation employed but must be regarded as reflex responses brought about through segmental and intersegmental reflex arcs including sympathetic neurons. The assumption that the sympathetic nerves in question include vasodilator fibers is supported by conclusive experimental data (Burn).

Stimulation of the receptors involved in cutaneo-visceral vasomotor reflexes probably is associated with changes in the tonic state of the cutaneous blood vessels. In our experiments prolonged application of warm packs resulted in localized cutaneous hyperemia which persisted for some time after removal of the stimulating agent.

During this interval cooling of the skin in the hyperemic area did not result in vasoconstriction in the gastrointestinal tract, nor did further application of a warm pack result in increased vasodilatation in the viscus. This is in full accord with the observation reported by Ruhmann<sup>4</sup> that local warming of the skin does not elicit reflex responses of the gastrointestinal musculature until dilatation of the cutaneous vessels in the stimulated area has taken place.

## 11250

**Response of Gonads and Gonaducts of Ambystoma Larvae to Treatment with Sex Hormones \***

CHARLES L. FOOTE (Introduced by Emil Witschi)

*From the Zoological Laboratory, State University of Iowa*

For many years *Ambystoma* has served as an experimental animal in studies of sex differentiation and sex development. Such studies have been made principally by means of parabiotic union of larvae or by gonad transplants. Since synthesized crystalline sex hormones are now available it seems of interest to determine the effects of these compounds upon the sexual development of the same species of salamanders and to compare the results of such experiments with those obtained by the above mentioned methods.

First reports on effects of crystalline sex hormones on sex differentiation in *Ambystoma* were made by Burns<sup>1, 2</sup> who concluded that injection of testosterone propionate into *Ambystoma punctatum* larvae causes genetic females to differentiate in a male direction, while estrone causes differentiation of genetic males in a female direction. Ackart and Leavy<sup>3</sup> obtained results similar to those of Burns with injections of estrone into *Ambystoma tigrinum* larvae.

The animals used in our study were larvae of *Ambystoma maculatum* of two races, a differentiated race from Georgia and a semi-differentiated race from Arkansas and a small lot of *Ambystoma tigrinum* larvae from Iowa. The sex hormones used were estrone (Theelin Parke-Davis Co.) estradiol dipropionate (Dioxocylin

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<sup>4</sup> Ruhmann, W., *München med. Wochschr.* 1933 **80**, 17.

\* Aided by grants from the National Research Council, Committee for Research in Problems of Sex.

<sup>1</sup> Burns, R. K., *Anat. Rec.* 1938 **71**, 447.

<sup>2</sup> Burns, R. K., *Anat. Rec.* 1939, **73**, 73.

<sup>3</sup> Ackart, R. I. and Leavy, S., *Proc. Soc. Exp. Biol. and Med.*, 1939 **42**, 720.

Ciba), and testosterone propionate (Perandren Ciba) † These hormones, dissolved in absolute ethyl alcohol, were added daily to aquarium water in concentrations of 500 gamma per liter The entire lot of animals was divided into three groups, one to receive testosterone treatment, a second to be treated with estrogens, and a third to serve as controls

The larvae of *Ambystoma maculatum* (Georgia) of differentiated race received hormonal treatment between the ages of 70 and 142 days *Ambystoma maculatum* (Arkansas) larvae of semi-differentiated race were treated when between 57 and 110 days old Hormonal treatment was stopped when these animals metamorphosed The tiger salamanders received treatment from the 50th day to the 221st day, the treatment being continued 70 to 100 days after metamorphosis At the time of first administration of hormone there were 50 control larvae, 70 animals to be treated with testosterone propionate, and 70 animals to receive estrogens Animals which died before the end of the experiment are included in data given in Table I

TABLE I.

Treatment	Females	Hermaphrodites	Males with cortex	Males
Controls	27	8	6	12
Testosterone	34	8	19	7
Estrogens	53	13	2	0

Some of the control males retain a vestige of cortex but otherwise possess typical testicular structure Some gonads of the larvae in the Arkansas group are of distinctly hermaphroditic character but must still be considered as genetic males (Witschi<sup>4</sup>) The gonoducts in control animals show no evidence of stimulation (Fig 1) The Wolffian ducts are developed to their full length in all larvae In *Ambystoma maculatum* only the anterior portion of the Mullerian ducts is present at the time of metamorphosis, but in the older *A. tigrinum* animals they reach the cloaca These ducts are of the neutral juvenal type as described by Rodgers and Risley<sup>5</sup> In no cases were the cloacal glands stimulated

The sex ratio in the group of larvae receiving testosterone propionate treatment remains unaltered (34 females and 34 males) This seems to indicate that the male hormone has little, if any, effect upon sex differentiation The ovaries seem to have been affected by the treatment in that there is no indication of the presence of an

† We wish to express our thanks to Ciba Pharmaceutical Products, Inc, and to Parke Davis Co for the generous supply of sex hormones

<sup>4</sup> Witschi, Emil, *J Exp Zool*, 1933, **65**, 215

<sup>5</sup> Rodgers, L T, and Risley, P L, *J Morph*, 1938, **63**, 119

ovarian cavity (Fig 2) In male sex glands one observes paradoxical feminization effects which will be discussed in a forthcoming paper In all animals of this group, regardless of genetic sex, there is an extreme stimulation of the Wolffian duct (Fig 2) and the cloacal glands The Wolffian and pronephric ducts are much convoluted, with crypts and pouches protruding from their walls, and in most cases the ducts are deeply pigmented and extend the full length of the body cavity The Mullerian ducts cannot be seen in the testosterone-treated animals This picture offered by the ducts is unequivocal proof that the testosterone is actually taken up from the water and produces characteristic effects even at very early stages of larval development

In estrogen-treated animals the sex ratio is definitely shifted in the female direction, with 53 females and 15 males These 15 "males", none of which possessed typical testes, are all found among the larvae which died relatively early They have hermaphroditic gonads with testicular and ovarian features of various proportions The ovaries of the 53 females resemble those of controls (Fig 3) It is statistically evident that these "females" are of two genetical types, true females and sex reversed males, though morphologically they cannot be separated While in metamorphosed animals of control groups the oviducts extend through the full length of the body cavity down to the cloaca, in estrogen-treated animals of same age they often end blindly before reaching the cloaca This inhibition in longitudinal growth is in contrast to the considerable inflation and consequent enlargement in diameter of these oviducts (Fig 3) At the larval stage only the upper parts of the oviducts, growing down from the pronephric region are present and exposed to hormonal influences These cranial parts are moderately enlarged in most cases The Wolffian ducts and cloacal glands retain a condition as in animals receiving no hormonal treatment The appearance of the oviducts is sufficient proof that like the testosterone the estrogenic hormones are also taken up by the larvae

*Conclusions* The presented data indicate that estrogens produce sex reversal in male salamanders while testosterone propionate exerts no corresponding influence upon genetical females As in adult animals the secondary sex characters respond also in the larval salamanders to the administration of sex hormones The female hormone stimulates slightly the larval Mullerian ducts, and the male hormone causes an extensive and very precocious stimulation of the larval Wolffian ducts and cloacal glands It is remarkable that the estrogens affect most profoundly the male gonads and the testosterone the male secondary sex characters The hormones do not induce



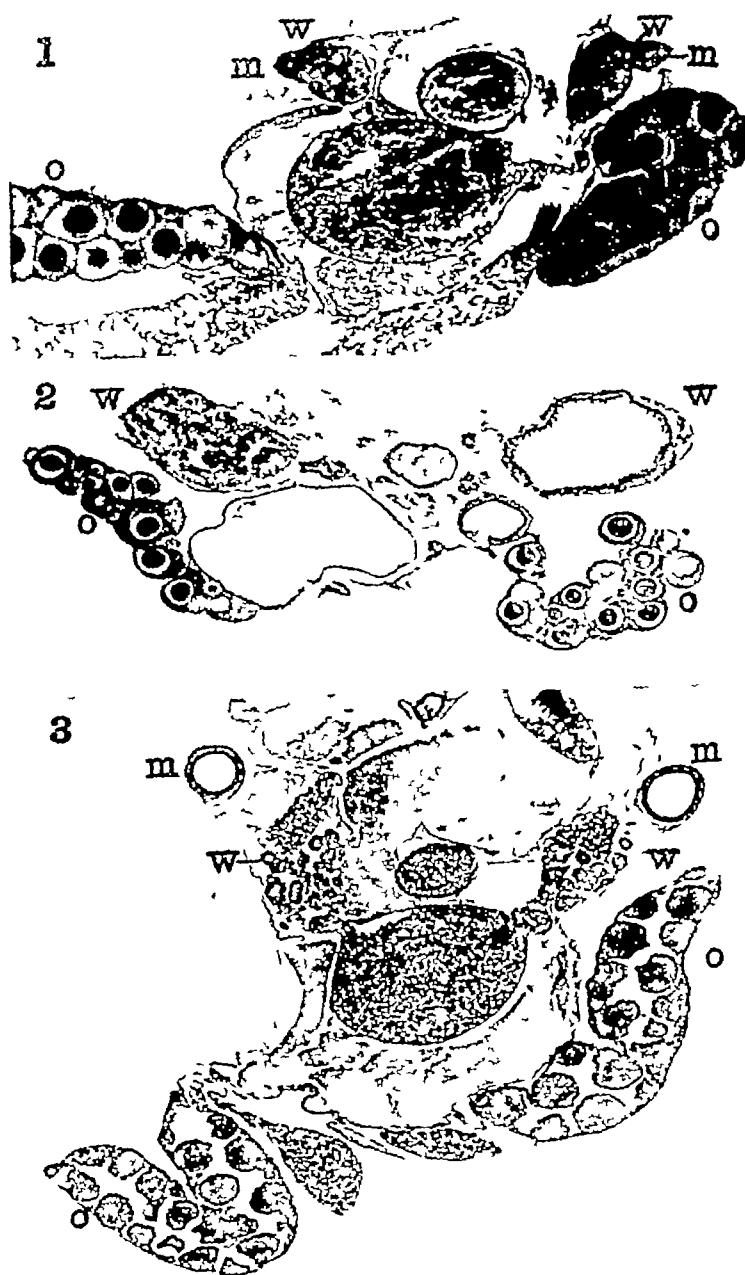


FIG 1

Cross section through the gonads and gonoducts of *A. tigrinum* control female. Note unstimulated Mullerian ducts (m) and Wolffian ducts (w).

FIG 2

Cross section through the gonads and gonoducts of *A. tigrinum* female treated with testosterone propionate for 171 days. Note absence of Mullerian ducts and stimulated Wolffian ducts (w) and close resemblance of the right duct (left in the picture) to the frog seminal vesicle.

FIG 3

Cross section through the gonads and gonoducts of *A. tigrinum* female treated with estradiol dipropionate and theelin for 171 days. Note stimulated Mullerian ducts (m) and unstimulated Wolffian ducts (w). All figures  $\times 25$ .

original formation of gonoducts they stimulate only the secondary (functional) enlargement of parts of ducts already present. It has been shown by means of parabiosis that the testes of males release some inductive substance which inhibits the development of the ovaries of female cotwins and indirectly may cause some genetic females to continue development in a male direction<sup>6</sup>. On the other hand in the male-female parabiotic combinations there is no precocious stimulation of either gonoducts or cloacal glands. These fundamental differences in the observed reactions prove that the crystalline sex hormones used in this experiment cannot be identical with the substances which normally act as inducers of sex differentiation.

11251

### Effect of Foods on Serum Esterase of Rats

J C FORBES, E L OUTHOUSE AND B E LEACH

From the Department of Biochemistry, Medical College of Virginia, Richmond, Va

In a previous publication<sup>1</sup> it was reported that the tributyrin splitting property (here called esterase concentration) of the serum of rats is markedly increased after poisoning with carbon tetrachloride and after feeding a high fat diet. On the other hand substances such as xanthine which exert a protective action on the liver against carbon tetrachloride will when subcutaneously injected into rats definitely lower the serum esterase concentration. Before proceeding further with the investigation into the possible significance of the serum esterase change in the animal's resistance to carbon tetra-

<sup>6</sup> Witte, Emil, *Illen & Sex and Internal Secretions*, Baltimore, 1930, 2nd ed., 145.

<sup>1</sup> Forbes, J C, and Outhouse, E L, *J Pharm and Exp Therap*, 1940, 68, 185.

chloride, it was necessary to study in greater detail the effect of various foods on the esterase concentration, laying particular emphasis on the changes resulting from the administration of single doses of fatty substances. In our previous recorded experiments the animals were kept on the high fat diet for a number of days and were allowed food up to the time of sacrificing.

The results herein presented deal with the effect of single doses of various foods on previously starved animals. They show that non-lipoid food stuffs, sucrose, glycerol and proteose-peptone exert no demonstrable effect on the serum esterase while neutral fats or fatty acids markedly increase it. No significant change was obtained in any case in the neutral fat, phospholipid or cholesterol content of the liver.

*Experimental* Rats weighing approximately 200 g, after being starved for the previous 24 hours were given the various supplements by stomach tube, except in the case of the Purina and Purina plus 40% butter fat diets. In these cases the required amount of food was fed early in the morning and only animals which consumed practically the whole amount in at least an hour and one-half were used for the various studies. The serum esterase, liver neutral fat, total phospholipid and cholesterol were determined by the methods previously described.<sup>1, 2</sup> The experimental results are shown in Tables I, II, and III.

It will be seen from the results recorded in Tables I, II and III that the administration of non-lipoid foods does not measurably affect the tributyrin-hydrolyzing power of the rat's serum. Glucose has also been used and found to be inactive. The administration of fats, oleic and palmitic acids, on the other hand, resulted in a marked rise in the serum esterase concentration. Since glycerol is inactive, while fatty acids have a pronounced effect, it would appear that the increase in esterase value is in some way related to an increase in some phase of fatty acid metabolism. The lack of apparent response following the feeding of Purina dog chow itself, which contains approximately 4% of ether extractable materials, indicates that a fair amount of fatty acids must be absorbed in order to obtain a definite response by the analytical method employed.

In a previous publication<sup>1</sup> it was shown that the serum esterase concentration of rats fed a high fat-low choline diet for several weeks, at which time a high fat content of the liver was produced, did not apparently differ from that obtained by feeding a high fat-Purina dog chow diet on which the deposition of fat in the liver was quite moderate. Other experiments in which sufficient choline was added to a high fat-low choline diet to prevent fat deposition in the

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<sup>2</sup> Outhouse, E. L., and Forbes, J. C., *J. Lab. and Clin. Med.*, in press

TABLE I  
Effect of Feeding Fat and Oleic Acid on the Serum Esterase and Liver Lipids of Rats

No of rats	Serum esterase			Liver, %			Remarks
	Max	Min	Avg	NF	P L	Chol	
4	25	14	20	0.9	4.13	0.25	Controls
4	31	17	25	2.0	4.06	0.28	Killed 1 hr after feeding by stomach tube 1 cc of butter fat per 100 g
5	34	18	30	1.5	4.19	0.27	" 2 " " " as above
2	13	30	31	1.0	4.08	0.29	" 3 " " " " "
5	16	28	33	1.2	4.18	0.26	" 4 " " " " "
2	25	20	22	0.8	3.83	0.25	" 5 " " " " "
3	28	21	25	2.0	4.23	0.27	" 6 " " " " "
2	24	22	23	1.1	3.78	0.28	" 7 " " " " "
2	23	21	22	2.2	4.30	0.27	" 8 " " " " "
1	-	19	19	1.1	4.77	0.24	Control
2	26	23	24	1.0	4.45	0.26	Killed 2 hr after feeding by stomach tube 1 cc of olive oil per 100 g
2	29	26	27	1.2	4.70	0.29	" 4 " " " " as above
2	17	22	34	1.7	4.78	0.29	" 6 " " " " "
1	37	26	31	1.7	4.58	0.25	" 8 " " " " "
2	28	26	27	-	-	-	10 hr 30 min after feeding as above
1	-	22	22	1.3	4.58	0.26	Control
1	13	28	30	1.6	4.38	0.26	Killed 1 hr after feeding by stomach tube 1 cc of oleic acid per 100 g
3	36	34	35	1.2	4.35	0.27	" 2 " " " " as above
3	44	38	41	1.7	4.24	0.25	" 4 " " " " "
3	44	38	41	1.4	4.20	0.25	" 6 " " " " "
2	35	28	31	-	-	-	Killed 3 hr 20 min after feeding by stomach tube 0.4 g sodium palmitate per 100 g

NF = Neutral fat plus cholesterol  
P L = Total phospholipids

TABLE II.  
Effect of Purina Dog Chow, and 60% Purina Plus 40% Butter Fat on the Serum Esterase and Liver Lipids of Rats

No of rats	Serum esterase			Liver, %			Remarks
	Max	Min	Avg	N.F	P.L	Chol	
4	24	15	19	1.5	3.89	0.29	Controls
5	24	15	19	1.2	3.89	0.28	Killed 2 hr 30 min after feeding 5 g Purina
5	23	15	18	1.1	3.58	0.27	" 4 " 30 " " " as above
5	24	13	18	1.3	3.73	0.27	" 6 " 30 " " " " "
3	16	16	16	1.4	3.76	0.25	" 8 " after feeding as above
4	22	17	19	0.9	3.78	0.29	Controls
2	34	32	33	1.1	3.85	0.31	Killed 2 hr 45 min after feeding 3 g of 60% Purina + 40% butter fat
2	32	32	32	1.4	3.18	0.25	Killed 4 hr after feeding as above
2	20	16	18	0.8	3.75	0.26	" 5 " " " " " "
4	24	14	20	1.6	3.37	0.27	" 7 " " " " " "
4	29	18	25	1.5	3.50	0.23	" 9 " " " " " "
2	20	10	15	2.2	3.16	0.25	" 25 " " " " " "
4	24	21	22	1.1	3.74	0.36	Controls
4	38	26	31	1.3	3.50	0.37	Killed 2 hr 45 min after feeding 5 g of 60% Purina + 40% butter fat
4	34	26	30	1.2	3.67	0.36	Killed 4 hr 45 min after feeding as above
4	34	34	34	—	—	—	" 5 " after feeding as above
5	27	23	25	1.1	3.56	0.35	" 7 " " " " " "
4	23	22	22	1.0	3.48	0.31	" 9 " " " " " "

N.F = Neutral fat plus cholesterol

P.L = Total phospholipids

TABLE III  
Effect of Feeding Non lipid Substance on the Serum Esterase and Liver Lipids of Rats

No of rats	Serum esterase			Liver, %			Remarks
	Max	Min	Avg	NF	PL	Chol	
1	—	—	14	1.3	3.00	0.28	Control
1	—	—	17	1.0	4.05	0.23	Killed 1 hr after 0.8 g of sucrose per 100 g by stomach tube
2	21	13	18	1.7	3.78	0.24	2 " " feeding as above
2	25	19	22	1.0	3.81	0.23	4 " " " "
2	22	16	19	1.4	3.42	0.22	6 " " " "
3	20	17	18	1.3	4.11	—	Controls
1	—	—	15	1.4	4.40	—	Killed 1 hr after 0.5 g glycerol per 100 g by stomach tube
1	23	12	18	1.6	4.01	—	2 " " feeding as above
1	20	13	15	2.0	4.02	—	4 " " " "
1	20	12	19	2.1	3.74	—	6 " " " "
2	16	16	16	—	3.70	—	7 " 20 min after feeding as above
1	—	—	20	0.9	4.00	0.29	Control
1	—	—	19	0.9	4.15	0.29	Killed 1 hr 30 min after 0.75 g protoseptone per 100 g by stomach tube
2	21	18	19	1.1	4.32	0.29	Killed 2 hr 30 min after feeding as above
2	25	25	25	1.0	4.20	0.29	4 " " " "
2	18	15	16	1.1	4.00	0.26	6 " " " "
NF = Neutral fat plus cholesterol PL = Total phospholipids							

N F = Neutral fat plus cholesterol  
P L = Total phospholipids

TABLE II  
Effect of Purina Dog Chow, and 60% Purina Plus 40% Butter Fat on the Serum Esterase and Liver Lipids of Rats

No of rats	Serum esterase				Liver, %		Remarks
	Max	Min	Avg	NF	PL	Chol	
4	24	15	19	1.5	3.89	0.29	Controls
5	24	15	19	1.2	3.89	0.28	Killed 2 hr 30 min after feeding 5 g Purina
6	23	15	18	1.1	3.58	0.27	" 4 " 30 " " " as above
5	24	13	18	1.3	3.73	0.27	" 6 " 30 " " " " "
3	16	16	16	1.4	3.76	0.25	" 8 " after feeding as above
4	22	17	19	0.9	3.78	0.29	Controls
2	34	32	33	1.1	3.85	0.31	Killed 2 hr 45 min after feeding 3 g of 60% Purina + 40% butter fat
2	32	32	32	1.4	3.18	0.25	Killed 4 hr after feeding as above
2	20	16	18	0.8	3.75	0.26	" 5 " " " " " "
4	24	14	20	1.6	3.37	0.27	" 7 " " " " " "
4	20	18	25	1.5	3.50	0.23	" 9 " " " " " "
2	20	10	15	2.2	3.16	0.25	" 25 " " " " " "
4	24	21	22	1.1	3.74	0.36	Controls
4	38	26	31	1.3	3.50	0.37	Killed 2 hr 45 min after feeding 5 g of 60% Purina + 40% butter fat
4	34	26	30	1.2	3.67	0.36	Killed 4 hr 45 min after feeding as above
4	34	34	34	—	—	—	" 5 " after feeding as above
5	27	23	25	1.1	3.56	0.35	" 7 " " " " " "
4	23	22	22	1.0	3.48	0.31	" 9 " " " " " "

NF = Neutral fat plus cholesterol  
PL = Total phospholipids

TABLE III  
Effect of Feeding Non lipid Substance on the Serum Esterase and Liver Lipids of Rats

No of rats	Serum esterase			Liver, %		Remarks
	Max	Min	Avg	NF	P L	
1	—	—	18	13	3.90	Control
1	—	—	17	10	4.05	Killed 1 hr after 0.8 g of sucrose per 100 g by stomach tube
2	21	15	18	17	3.78	" 2 " " feeding as above
2	25	19	22	10	3.81	" 4 " " " " "
2	22	16	19	14	3.42	" 6 " " " " "
3	20	17	18	13	4.11	Controls
1	—	—	15	14	4.40	Killed 1 hr after 0.5 g glycerol per 100 g by stomach tube
4	23	12	18	16	4.04	" 2 " " feeding as above
4	20	13	15	20	4.02	" 4 " " " " "
1	26	12	19	21	3.74	" 6 " " " " "
2	16	16	16	—	3.70	" 7 " 20 min after feeding as above
1	—	—	20	0.9	4.00	Control
1	—	—	19	0.9	4.15	Killed 1 hr 30 min after 0.75 g proteoseptone per 100 g by stomach tube
2	21	18	19	1.1	4.32	Killed 2 hr 30 min after feeding as above
2	25	25	25	1.0	4.20	" 4 " " " " "
2	18	15	16	1.1	4.00	" 6 " " " " "

NF = Neutral fat plus cholesterol  
P L = Total phospholipids



liver have been found not to affect the esterase response. A lack of correlation between the degree of visible lipemia and the esterase value has been noticed. In many cases the lipemia would have practically disappeared while the esterase value still remained high, while in others a marked lipemia was associated with a relatively low esterase value.

In considering the results herein presented it should be realized that only changes in the tributyrin hydrolyzing power of the serum are under consideration. Mosters<sup>3</sup> did not find any definite correlation between the ability of blood to hydrolyze methyl butyrate and tributyrin. He found that ascorbic acid administration increased the blood's power to hydrolyze methyl butyrate but the effect on tributyrin hydrolysis was very indefinite, the two effects being sometimes in opposite directions. Gajdos,<sup>4</sup> however, found the subcutaneous administration of ascorbic acid to invariably increase the serum's power to hydrolyze either substrate.

Any discussion as to the possible significance of serum esterase in fat metabolism and in determining an animal's resistance or susceptibility to halogen hydrocarbons seems unjustifiable at the present time. However, further data is being accumulated in an attempt to definitely determine the significance of this enzyme in these conditions.

*Summary* It has been shown that the oral administration of non-lipoid foods, glycerol, sucrose, glucose and proteose-peptone exert no demonstrable effect on the serum esterase of rats. The administration of neutral fats, oleic and palmitic acids leads to a marked increase which lasts for several hours.

The authors gratefully acknowledge their great indebtedness to the John and Mary R. Markle Foundation for financial assistance in this research.

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<sup>3</sup> Mosters, J., *Klin. Wochschr.*, 1936, **15**, 1557

<sup>4</sup> Gajdos, A., *Compt. rend. soc. biol.*, 1930, **131**, 59

## 11252 P

Preparation of Antigenic Material Inducing Leucopenia from  
*Eberthella typhosa* Cultured in a Synthetic Medium

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Substances presumably free from whole protein which exhibit antigenic and toxic properties have been derived from cultures of the *Salmonella* group of organisms by Borvin and his associates,<sup>1</sup> and Topley and his coworkers.<sup>2</sup> The exact chemical nature of such agents as well as several of their immunologic aspects and effects on the animal body await further investigation. The present communication describes the occurrence of certain phenomena following the injection of mice and rabbits with a toxic, antigenic material prepared from cultures of *Eberthella typhosa* grown in a synthetic medium. The use of a medium containing ingredients which could all be removed by dialysis obviously should facilitate the separation of bacterial products from constituents of more complex media of unknown composition.

Adopting as a base the inorganic salts suggested by Burrows,<sup>3</sup> a synthetic medium was devised of the following composition:  $(\text{NH}_4)_2\text{SO}_4$ , 10 g, NaCl, 1 g,  $\text{K}_2\text{HPO}_4$ , 4 g, glucose, 7 g, acid hydrolyzed casein 12 g, tryptophane, 0.2 g, cystine, 0.4 g, phenol red (0.2% solution), 10 cc, and distilled water, 2,000 cc. The reaction was adjusted to pH 7.4 and the mixture sterilized in the autoclave, except for the glucose, which was later added as a sterile 50% solution.

A recently isolated strain of *E. typhosa* possessing the characteristics of the smooth form was employed.

**Method.** 14 flasks each containing 2,000 cc of the solution were inoculated with 15 cc of a 12-hour culture of *E. typhosa* that had been cultivated in this same medium for 3 successive transfers. These flasks were incubated at 37°C. At 8-hour intervals, 10 cc of a 50% solution of glucose were added and sufficient NaOH to restore the pH to approximately 7.4. After 72 hours 10 g of sodium acetate

\* DeLamar Fellow

<sup>1</sup> Borvin, A., Mesrobian, I., and Mesrobian, L., *Compt rend Soc d Biol* 1933, **113**, 490<sup>2</sup> Raistrick, H., and Topley, W. W. C., *Brit J Exp Path* 1934, **15**, 113<sup>3</sup> Burrows, W., *J Inf Dis* 1939, **64**, 145

and 3 volumes of 95% alcohol were introduced into each flask which was then placed in the refrigerator for 48 hours. The supernatant fluid was decanted and the precipitate of cells and a grey, gummy material was recovered by centrifugation. These sediments from all the flasks were then combined by taking them up in 1,000 cc distilled water. The emulsion was shaken with glass beads for 2 hours at 37°C, placed in the refrigerator overnight and again shaken for 2 hours. After centrifugation, the solution was retained and the insoluble material resuspended in 300 cc of distilled water and the process repeated. The combined aqueous extracts, freed from particulate matter by centrifugation for ½ hour at 4,000 to 5,000 rpm, were placed in cellophane bags and dialyzed against running tap water at 10-14°C for 24 hours. Sodium acetate (0.5%) was added to the opalescent suspension, the reaction was made just acid to litmus with acetic acid and 3 volumes of alcohol added. After standing over night in the refrigerator, the precipitate was removed, resuspended in water and reprecipitated with 3 volumes of alcohol. This procedure was twice repeated. After the last precipitation the deposit was dissolved in 200 cc distilled water and shaken twice with 40 cc of chloroform and 8 cc of n-butyl alcohol according to the method of Sevag<sup>4</sup> to remove any protein present. Following the addition of 3 volumes of alcohol, the precipitate was washed twice with 70% alcohol and stirred with 100 cc of distilled water to give a light grey, colloidal suspension with a pH of 6.9. The yield was 0.8 g as determined by drying and weighing an aliquot of this solution.

Qualitative tests showed an absence of protein by the sulfosalicylic acid and HNO<sub>3</sub> tests. The biuret and ninhydrin tests were faintly positive. Millon and Hopkins-Cole tests were negative. A strongly positive Molisch reaction was obtained. After acid hydrolysis treatment with phenylhydrazine yielded osazone crystals which were similar to those obtained with glucose. Elementary analyses gave total N, 7%, amino N, 1% total P 5.6%. After hydrolysis with 1 N HCl for 1½ hours at 100°C, the product yielded 3.2% amino N and 13.4% reducing sugar (calculated as glucose). The ash was 24.4%.

*Antigenic properties.* With 3 typhoid antisera the colloidal suspension induced a precipitate in a dilution of 1:200,000 (ring test 2 hours at 20°C). To 3 normal rabbits a total of 15 mg of this antigen in increasing doses were administered intravenously over a period of 3 weeks. Their sera exhibited agglutination titers of 1:1280 with a suspension of strain 0901 of *E. typhosa* and precipitin reactions with

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<sup>4</sup> Sevag, M. G., *Biochem. Z.*, 1934, **273**, 419

TABLE I.  
Rabbit No 5

Hours after injection	Body temperature	Total WBC count
0	102.4°F	11,750
0.15 mg antigen inj intravenously		
2	106.2	2,600
4	106.6	2,350
8	104.8	11,000
24	104.0	21,150
32	103.6	14,000

the suspension of the antigen were positive in a dilution of 1:1,000,000 of the latter

**Toxicity** 4-5 mg of the preparation injected intraperitoneally into mice weighing 15-18 g killed 90% of the animals. Five rabbits of 1.5 to 2.5 kg body weight died within a few hours after intravenous injections of 0.4-0.5 mg. Intravenous administration of 0.1-0.25 mg was followed by marked leucopenia and fever in rabbits within 1 to 2 hours, subsequently attended by leucocytosis and a drop in temperature. Table I presents the results obtained in one of 10 rabbits so treated, all of which exhibited similar reactions.

Differential counts showed that the leucopenia resulted from the almost complete disappearance of the polymorphonuclear cells from the circulating blood. During the subsequent leucocytosis, these cells rose to a high level and many of them were found to be immature as indicated by the presence of single or double lobed nuclei. A number of years ago a leucopenia in rabbits was described by Zinsser and Tsen<sup>5</sup> following large intravenous doses of suspensions of typhoid bacilli. While the present work was in progress, Smith<sup>6</sup> and Dennis and Senekjian<sup>7</sup> reported the isolation of leucocidal materials from cultures of *E. typhosa*. The material studied seems to differ from the leucocidal factor recently isolated by the latter authors in that it is precipitable from an acidified aqueous solution by the addition of alcohol. Twenty-four hours following the intracutaneous injection of 1 mg of the material into normal and immune rabbits, severe inflammatory reactions 2.5 cm in diameter appeared characterized by redness and marked edema.

The toxic properties of our substance appeared to be thermostable, since boiling for 1/2 hour did not alter its capacity to kill rabbits or to induce a leucopenia and fever. A study of the relationship between its toxic and immunologic characteristics is in progress.

**Summary** An antigenic substance has been isolated from cul-

<sup>5</sup> Zinsser, H., and Tsen, E, *J. Immunol.*, 1916, **2**, 247.

<sup>6</sup> Smith, E. V., *Am. J. Hyg.*, 1939, **29** (section B), 15.

<sup>7</sup> Dennis, E. W., and Senekjian, H., *Am. J. Hygiene*, 1939 **30** (section B), 103.

tures of *E typhosa* grown in a synthetic medium. Appropriate amounts kill mice and rabbits and smaller quantities induce a leucopenia and fever in rabbits.

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## 11253

### Skin Sensitivity of Man to Bovine Plasma and Its Albumin and Globulin Fractions

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The injection of whole bovine plasma in man was found to be associated with a considerable number of untoward reactions<sup>1</sup>. In an effort to determine the source of these reactions the whole plasma was fractionated into albumin and globulin portions. A study of skin sensitivity of man to these materials forms the basis of this paper.

*Method* From sterile bovine plasma prepared as described elsewhere,<sup>1</sup> albumin and globulin fractions were separated by ammonium sulfate precipitation. The details of this procedure will appear in a subsequent publication.

One hundred and forty-six patients selected at random\* were tested for skin sensitivity to bovine albumin solution, bovine globulin solution, and whole bovine plasma. Normal NaCl solution was used as a control. Five-hundredths cc of each of the above was injected intradermally and readings were taken after 20 to 30 minutes. No change or disappearance of the original wheal was considered negative, slight enlargement of the wheal with a small area of erythema 1+, a marked halo of erythema without pseudopodia 2+, pseudopodia and enlargement of the original wheal to twice original size

<sup>1</sup> Wangenstein, O H., Hall, Harry, Kremen, A. J., and Stevens, B., in press.

\* Acknowledgment is made to Dr Macnider Wetherby, Head of the Outpatient Department of the University of Minnesota Hospitals for his cooperation in this study.

TABLE I  
Results of Skin Tests

Solution used	Reactions									
	0		1+		2+		3+		4+	
	No	%	No	%	No	%	No	%	No	%
Saline solution	136	93	7	5	3	2				
Bovine albumin solution	99	68	39	27	8	5				
" globulin "	56	38	50	35	36	25	3	2	1	0.7
Whole bovine plasma	25	17	43	30	53	36	23	15.5	2	1.5

TABLE II  
Relationship of Type of Reaction with Albumin and Globulin Fractions to Type of Reaction with Whole Plasma

Plasma Reaction	Type of albumin reaction Figures expressed in %			Type of globulin reaction Figures expressed in %			
	0	1+	2+	0	1+	2+	4+
0	100			100			
1+	100			63	37		
2+	42	49	9	35	50	15	
3+	35	50	15	20	75	5	
4+		50	50		50		50

3+, and marked edema, induration, pseudopodia and erythema 4+

*Results* The results of skin tests on 146 patients are tabulated in Table I. It is noted that a wide difference of skin sensitivity appears between the albumin, globulin and whole plasma. There were 7% positive reactions (1+ to 2+) with the control saline solution. The albumin caused the least number of positive reactions, the globulin was intermediate and the whole plasma caused the largest number of positive reactions as well as those of greatest magnitude.

Table II shows the relationship of the reactions of the albumin and globulin fractions to those of whole plasma. The results are tabulated as the percentage of the type of reaction of the plasma fractions that occurred with the different types of reaction to whole plasma. In no instance did the type of reaction of the albumin or globulin fraction exceed that of the whole plasma in intensity. In only 4 cases (2.7%) was the reaction of the albumin equal to that of the plasma and in 34 cases (23.3%) was the reaction of the globulin fraction equal to that of the whole plasma.

*Summary and Conclusions* 1 The skin sensitivity of man to bovine plasma and to its albumin and globulin fractions has been investigated. 2 The skin sensitivity of man to the three preparations tested is least marked with the albumin fraction of bovine plasma.

## Evacuation of the Gall Bladder in Patients with Pernicious Anemia \*

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This is the third of a series of studies analyzing the rate of emptying of the gall bladder in patients with lesions of the stomach.

In the first series<sup>1</sup> it was observed that peptic ulcer patients displayed a significantly faster rate of emptying than controls of comparable age. Since the mean curve of emptying for this group could be virtually reproduced in normal individuals merely by injecting one egg-yolk directly into the duodenum (Fig 1, left) and since the initial discharge of food into the duodenum is known to be more rapid in individuals having gastric and duodenal ulcer, the faster rate of emptying of the gall bladder in such patients was attributed to a greater food stimulus rather than to an increased production of gastric juice.

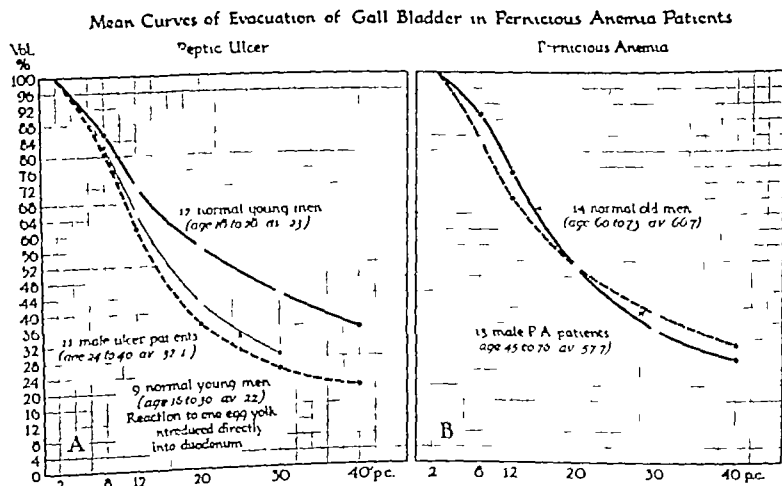


FIG 1

Mean curves of the evacuation of the gall bladder in patients with lesions of the stomach. Ordinates, percentage volume of bile in the gall bladder, abscissae, minutes after a standard meal.

\* Aided by grants from the Medical Research Fund of the Graduate School

<sup>1</sup> Boyden, E A, and Berman, T M, *Radiol*, 1937 **28**, 273

This interpretation was strengthened by the second study.<sup>2</sup> This dealt with patients having carcinoma of the stomach. In this group, marked reduction of the amount of free HCl failed to retard the emptying of the gall bladder.

The present report is based upon cholecystographic studies of 22 consecutive, unselected male patients for whom a clinical as well as hematological diagnosis of pernicious anemia had been established. All of the patients were receiving treatment with liver extract. The hemoglobin content of the blood had either reached normal levels or was responding to treatment. The histamine test had disclosed complete absence of free acid in all patients, and only minimal amounts of total acids.

Of special interest was the fact that the gall bladder could not be visualized in 40% of these individuals, notwithstanding the use of the intravenous method of introducing the dye and the absence of any history suggesting gall bladder disease. In the other groups visualization had failed in only 9% of ulcer patients and in 22.7% of those with carcinoma.

In the remaining 13 of the 22 patients, the mean curve of emptying of the gall bladder approximated that of the controls (Fig. 1, right), being a little faster in the first 20 minutes after a standard meal† and a little slower in the last 20 minutes, but not significantly so.

Since there was complete absence of free HCl in all 22 patients, even after stimulation by histamine, one must conclude that free HCl in the stomach is not an essential factor in the evacuation of the human gall bladder.

The final report will include a comparable study of female patients.

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<sup>2</sup> Ritchie, W. P., and Boyden, E. A., *Proc. Soc. Exp. Biol. and Med.*, 1937, **36**, 815.

† The modified Boyden meal, consisting of 4 egg yolks mixed with an equal amount of milk to which a pinch of sugar and a drop of vanilla extract has been added.



Poliomyelitis in *Sigmodon hispidus littoralis* Rats \*

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Recently, Armstrong<sup>1</sup> reported that he produced poliomyelitis in *Sigmodon hispidus hispidus* rats (Eastern cotton rats) by injecting poliomyelitis virus (Lansing strain). He was unsuccessful when other strains were used. It is almost impossible to distinguish this animal from *Sigmodon hispidus littoralis*, an animal more common in the South and relatively inexpensive.

Ten rats (*Sigmodon hispidus littoralis*) were given a 10% suspension of Flexner's M V strain, 5 getting purified and eluted virus and 5 unpurified virus. Armstrong's technique was followed, the doses in each instance in the following experiments being 0.06 cc intracerebrally, 0.06 cc intranasally and 0.5 cc subcutaneously. Two of the animals which received unpurified virus died 5 days later. Pathologically, the central nervous system showed nothing that resembled poliomyelitis. A suspension of the brain and cord obtained from these animals was injected into 5 rats. These remained normal after nearly 3 months of observation.

Nine virus strains [Flexner's M V, Toomey, Flexner (Phil, Wolman), Kramer, Harmon, Howitt, Trask W E, Flexner W E, Australian] were each injected into 5 rats. One rat given Flexner M V virus died the second day after injection. 1 given Flexner (Phil) virus died the sixteenth day, 1 Kramer the sixth day, 1 Harmon, the second day, 1 Harmon, the ninth day, 1 Howitt, the fifth day, 1 Flexner W E, the fourth day, 1 Australian the fifth day. There was nothing found in any of the cord sections from these animals that resembled experimental poliomyelitis. The animals that died during the first few days after injection had some local intracerebral hemorrhage. Their deaths were probably due to trauma.

After these 8 animals died the cord and brain of each were emulsified and injected into 3 rats (24 in all). After 2 months of observation the animals were still alive.

Nearly 3 months after the original experiment with the Flexner M V virus strain or 2 months after injecting the other 9 poliomyelitis strains, 59 of the 74 surviving rats were injected with Arm-

\* Aided by a grant from The National Foundation for Infantile Paralysis Inc.  
1 Armstrong, Charles, *Pub Health Rep*, 1939 **54**, 1719

strong's Lansing virus strain. Concomitantly, 10 rats never previously injected were used as controls. The animals began to die within 4 days after the injection. Every animal but one of the 69 succumbed within 10 days. The rats showed all types of paralyses, the most common one being bulbar. There were some which had incoordination and peculiar rolling or circling movements as well. The pathologic findings were the same as those described by Lillie and Armstrong.<sup>2</sup> However, there was not much perivascular infiltration.

Armstrong's virus (Lansing) was carried through 5 generations of rats in only 22 days, transmission being made every fourth day.

*Conclusion.* The Lansing strain of virus isolated by Armstrong easily produces disease in *Sigmodon hispidus littoralis* rats. Thus far, we have been unable to produce poliomyelitis with any of the 9 stock virus strains carried in our laboratory and named in this paper.

## 11256 P

### Quantitative Alteration in Renal Corpuscles of Salamanders Through Variation in Hypophyseal Tissue \*

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Previous reports have shown that increasing the amount of hypophyseal tissue in an amphibian embryo by grafting gives rise to an increase in the thickness of the basement membrane of the renal glomerulus.<sup>1</sup> It has been pointed out that this thickening is similar in appearance to the thickened membrane in human cases of hypertension.<sup>1, 2</sup> The size of the heart, vasoconstriction, reduced heart rate and other conditions in these test animals simulate a hypertensive state.<sup>3</sup> The hypophysectomized individuals show the opposite results of hypotension and may now be stated to have correlated with this a thin basement membrane.

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<sup>2</sup> Lillie, R. D., and Armstrong, Charles. *Pub. Health Rep.* 1940, 55, 115.

\* Aided by a grant from the research funds of the Graduate School, University of Minnesota.

<sup>1</sup> Blount, Raymond F., *Anat. Rec.*, 1936, 65, 1.

<sup>2</sup> McGregor, Leone, *Am. J. Path.*, 1930, 6, 347.

<sup>3</sup> Blount, Raymond F., *J. Exp. Zool.* 1935, 71, 421.

The arrangement of the loops of the capillary tuft indicates that there is a simplification in these hypertensive glomeruli<sup>4</sup>. This is stated by McGregor<sup>2</sup> to be true in human hypertension. She also states that there is contraction of glomeruli although no measurements or volume determinations were made. Goormaghtigh<sup>5</sup> mentions contraction of glomeruli in ischemic kidneys.

In our test animals there appeared to be a contraction in many glomeruli with an increase in the capsular space. How much this increase in space is due to a shrinkage of the glomerulus and how much to an increase in capsular volume had to be determined. The present observations deal with quantitative determinations of volume for the total renal corpuscle, the glomerulus and the capsular space of hypophysectomized, control, and triple pituitary animals. This has been done by the paper cut-out method using every serial section. Two groups of animals were employed. In one, values for every fifth corpuscle were computed individually in 3 sets each consisting of an hypophysectomized, a control and a triple pituitary animal. In all there were 153 corpuscles for which computations were made. In the other series the volumes for all the corpuscles were determined and the averages obtained for the 12 animals of 4 sets.

The general magnitude of one of these glomeruli is about 0.00150 cu mm. However, considerable variation was found in the volume since there were extremes of 0.00011 and 0.00880 cu mm. The larger glomeruli were at the cranial pole of the kidney and the smaller ones at the caudal. Although the smallest glomeruli were found in the triple pituitary animals some few were large. The cranio-caudal gradation in glomerular size was not a factor in the differences in the test animals since the distribution of glomeruli, as to myotome level, was about the same in all 3 types. There was no definite relationship between body size and glomerular size. However, the amount of body volume per glomerulus was greater in the larger animals.

The percentage relationship that the average glomerulus bears to the entire corpuscle is less in the triple pituitary than in the control animal in all the cases except one which is mediocre by other criteria. This animal follows the trend if the mode rather than averages is used. In the hypophysectomized individuals the glomerulus occupies a greater proportion of the capsule in all but 2 cases, the above set and one other. Some glomeruli of these animals show enlargement due to vasodilation. The percentages, in the 4 sets on which values

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<sup>4</sup> Blount, Raymond F, *Anat Rec*, 1937, **67**, Suppl. No. 3, 7

<sup>5</sup> Goormaghtigh, N, *Proc Soc Exp Biol and Med*, 1939, **42**, 688

were obtained for all glomeruli, are in the hypophysectomized control and triple pituitary animals respectively as follows 79, 74, 49, 81, 65, 39, 75, 68, 61, and 76, 67, 51. In 2 of these 4 sets the relatively larger capsular space in the triple pituitary animal results from the glomerulus being contracted and in the other 2 from the great increase in the volume of the capsule. Stasis is probably responsible for the large size of some of the triple pituitary glomeruli. In all cases the generally thickened membrane (up to 8 times normal) balances to some extent the contraction of the capillary tuft in determining the glomerular volume.

These observations show that the renal corpuscle of animals with an increased amount of pituitary tissue have glomeruli that often but not always exhibit contraction, and that there is a clear-cut reduction in the relative size of these glomeruli. In the hypophysectomized individuals the corpuscles show no uniform alteration in glomerular size but a definite tendency toward a reduction in the relative size of the capsular space.

## 11257 P

### Parasympathomimetic Effects of Erythrocytes in the Cat

M. B. BENDER\* (Introduced by Gregory Schwartzman)

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Using the completely denervated iris of the cat as an indicator of circulating autonomic substances, it was found that constriction of the pupil ensued from 35 to 70 seconds following the intravenous administration of 10 cc of whole human blood. The blood of rabbit, guinea pig, mouse, monkey, hog and hemolyzed erythrocytes of cat also produced a constriction of the completely denervated iris of the cat. Identical amounts of blood from dog, sheep and ox were without significant effect on the same preparation. The most conspicuous reactions were obtained with rabbit's blood. Large quantities of dog, ox, or sheep blood sometimes produced a slight and slow constriction of the cat's denervated iris.

Other effects caused by the intravenous injection of blood into the cat were a fall in blood pressure, as shown by Abramson, *et al.*,<sup>1</sup> and

\* This investigation has been aided by grants of the Josiah Macy, Jr., Foundation and the Dazian Foundation for Medical Research.

<sup>1</sup> Abramson, D. L., Wasserman, P., and Senior, F. A., *Am. J. Physiol.*, 1935, 124, 402.

Fuzii,<sup>2</sup> decrease in heart rate, relaxation of anal and urinary sphincters and hypersalivation, all parasympathomimetic manifestations

The vaso depressor and miotic properties of the blood were located chiefly in the erythrocytes. Laked red blood cells also exhibited these properties. Intravenous injection of serum never produced a visible change in the iris.

The washed red blood cells caused a pupillary constriction from 35 to 80 seconds after their intravenous injection whereas the laked erythrocytes produced a much quicker response, the miosis appearing from 12 to 30 seconds after the administration. The rabbit's erythrocytes showed two types of depressor effects on the cat's blood pressure, an early transient drop from 6 to 10 seconds after the injection of the cells and a later prolonged fall from 45 to 70 seconds after the administration. The hemolyzed red blood cells showed only the early depressor effect. The findings concerning the effect upon blood pressure were similar to those of Fuzii.<sup>2</sup>

Furthermore, the cat's own laked blood produced an early and transient drop in its blood pressure, and a constriction of its completely denervated iris. The smallest quantity of rabbit's blood necessary to produce a constriction of the denervated iris was 0.5 cc of one percent saline suspension of erythrocytes per kilo of body weight of cat. The intact cells were more effective in producing parasympathomimetic reactions than the laked erythrocytes. By centrifuging the hemolyzed red blood cells of the rabbit, it was found that the constrictor factor of the blood was also present in the supernatant red fluid.

Since the iris was completely denervated, the miotic action of the injected bloods mentioned must be due to a chemical agent. The substance had no effect on denervated facial striated muscle or on the radial smooth muscle of the iris denervated by the removal of the superior cervical ganglion. The constriction of the pupil by the intravenous injection of erythrocytes was demonstrated in the eye in which either the oculomotor nerves (preganglionic) or ciliary nerves (post-ganglionic) were acutely sectioned. Atropine sulfate did not hinder the miosis produced by larger quantities of blood. Eserine did not seem to augment the reaction.

Intravenous injections of whole blood, erythrocytes, or laked corpuscles of the species mentioned produced no reaction in the completely denervated iris of the monkey.

I am indebted to Dr. Gregory Schwartzman for his valuable aid and suggestions in this problem.

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<sup>2</sup> Fuzii, K., *Tohoku J. Exp. Med.*, 1939, **35**, 384.

## 11258 P

Detection of Human Influenza Virus in Throat Washings by Immunity Response in Syrian Hamster (*Cricetus auratus*)

R M TAYLOR (Introduced by J H Bauer)

From the Laboratories of the International Health Division of the Rockefeller Foundation New York

In a previous communication<sup>1</sup> the serial passage of the human influenzal virus in the European hamster (*Cricetus cricetus*) was reported. While this hamster was shown to be susceptible to a recently isolated virus strain it was not demonstrated because of lack of material at the time, whether or not infection could be transferred directly from humans.

This present report deals with the infection of the Syrian hamster (*Cricetus auratus*) from throat-washings of persons ill with epidemic influenza.

**Material** The washings were collected in buffered broth during the early months of 1939 from persons acutely ill with influenza. The presence of virus originally in these specimens had been demonstrated by the inoculation of ferrets.<sup>2</sup> The washings had been preserved for 9 to 10 months in a low-temperature cabinet at  $-76^{\circ}\text{C}$  when the inoculation of the hamsters was made. The hamsters employed were from 3 to 5 months of age and weighed approximately 100 g each.

**Method** Preceding inoculation, 1 cc of blood was withdrawn by cardiac puncture from each hamster. From 0.3 to 0.4 cc of unfiltered throat-washing was administered intranasally while the animal was under light ether-anesthesia. Twelve to 14 days after the intranasal inoculation a second blood sample was taken. The blood serum obtained before and following inoculation was then titrated for neutralizing antibodies against the PR8 strain of influenzal virus.

**Results** So far, 4 preserved washings obtained during the 1939 epidemic, but from separate localities, have been tested. All of these specimens were known to contain virus originally.

None of the blood samples taken before inoculation showed any neutralizing effect upon the virus in a 1:2 serum-mixture, but samples taken 12 to 14 days after inoculation of the hamsters with each of these 4 washings all neutralized 1000 MLD of the virus in dilu-

<sup>1</sup> Taylor, R. M., and Dreguss, M., *Proc. Soc. Exp. Biol. and Med.*, 1940, **43**, 100.

<sup>2</sup> Horsfall, F. L., Jr., Hahn, R. G., and Richard, F. R., *J. Clin. Inv.* 1940, **19**, 379.

tions above 1:32. Despite the immune response, these animals manifested no symptoms or gross pathological lesions which could be used as diagnostic criteria.

Three washings which did not contain influenzal virus as shown by ferret-inoculation failed to stimulate neutralizing antibodies in the hamster. There appears to be little doubt, therefore, that the immune response in the hamster was specific and resulted from influenza-virus infection. Moreover, the infection established through the inoculation of throat-washings may be transferred from one hamster to another.

Hitherto the ferret has been used almost exclusively for the detection of the virus in human material, either by immunity response or by subsequent passage and identification in mice. The virus has been obtained directly in white mice from human throat-washings,<sup>3</sup> but this is a tedious and unreliable method. Several mouse-passages are required before the virus becomes manifest, and, in the experience of the author, only a small proportion of strains which may be identified in throat-washings by means of the ferret can be obtained by direct inoculation of mice. Nor has it been possible in this laboratory to demonstrate the development of immunity in mice inoculated with washings known to contain the virus.

Thus the hamster is the only animal other than the ferret in which it has been shown that the presence of virus in human throat-washings may be detected by the immunity response to the original inoculum. The relatively low cost of the hamster, the ease with which it may be bred in the laboratory, and its apparent resistance to canine distemper and other epizootics to which the ferret is so subject would make it a welcome substitute for the ferret. However, it is realized that these observations must be extended before it can be determined to what degree the hamster may be used as an adjunct or substitute for the ferret in the study of human influenza.

It may be added that 3 of the throat-washings which produced an immunity-response in the hamsters were at the same time administered intranasally to cotton rats (*Sigmodon hispidus hispidus*). Two of the rats showed no rise in antibodies, and the third gave only a minimal and questionable response.

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<sup>3</sup> Francis, T., Jr., and Magill, T. P., *Proc. Soc. Exp. Biol. and Med.*, 1937, **36**, 132.

## 11259 P

Growth and Differentiation of *Daphnia Magna* Eggs *in vitro*

VASII OBRESHKOVE AND ALAN W FRASER

*From Bard College, Columbia University*

In the course of some ultra-centrifuging experiments there arose the need of rearing the parthenogenetic eggs of *Cladocera in vitro*. A review of the literature revealed no record of the successful growth of eggs of this group of animals outside the brood-chamber of the mother. We have succeeded in rearing the eggs of *Daphnia magna* in tissue culture slides and inasmuch as the embryos are transparent, this enabled us to make observations on some of the sequence of events in the course of the development of the embryos without the necessity of staining and sectioning.

A number of different media were used in an endeavor to grow *Daphnia magna* eggs *in vitro*. A modified Ringer's solution utilized successfully for physiological work on isolated organs of *Cladocera* by Levy<sup>1</sup> proved injurious to the eggs. The growth of eggs in a medium consisting of the fluid obtained from the brood-chamber of animals in which there were developing eggs showed no advantage over a simpler medium consisting of sterile pond water. This is of considerable interest in view of the fact that it has been held (Dearborn,<sup>2</sup> Birge<sup>3</sup>) that the brood-chamber of *Cladocera* secretes a fluid which serves as nourishment in the course of the development of the embryos. Careful controlled experiments revealed that the parthenogenetic eggs in the brood-chamber of *Daphnia magna* completed their development in about 46 hours at 25°C. When the parthenogenetic eggs of this animal were reared in sterile pond water on tissue culture slides, they too completed their embryonic history from eggs to free swimming independent organisms in the same period of time. This demonstrates very clearly that the eggs of this animal are self-sufficient with regard to the nutritive material already stored in them at the time of their deposition.

A *Daphnia magna* female the eggs of which were to be removed from the brood-chamber, was placed in a depression slide with round polished cavity 15 mm in diameter and 3 mm deep filled with sterile

<sup>1</sup> Levy, R, *Compt Rend Soc Biol*, 1927, **97**, 1600

<sup>2</sup> Dearborn, G V N, Chapter on protoplasm and simple animal functions *Human Physiology*, Philadelphia and New York, Lea & Febiger, 1909

<sup>3</sup> Birge, E A, Chapter on water fleas, *Fresh Water Biology*, Ward and Whipple, New York, John Wiley & Son, 1918



pond water With the aid of 2 fine needles the animal was held in place in the field of the binocular microscope and the ventral edges of the carapace were carefully spread apart By gently moving the body of the animal back and forth with the needles, the eggs were made to roll out of the chamber without being subjected to any pressure The eggs were transferred to a new depression slide containing sterile pond water and were incubated at 25°C The chorion and the vitelline membranes of the eggs are very easily injured The removal of eggs from the brood-chamber of the mother without injury to them determines the degree of success of rearing the eggs of this animal *in vitro*

We have obtained several complete series of photomicrographs of *Daphnia magna* eggs taken at 3-hour intervals throughout the course of their development *in vitro* We have made observations on some of the more prominent changes in the course of the development of the living material as observed by gross microscopical examination The first sign of movement of the body was observed in embryos 30 hours old At this stage also the heart exhibited its first pulsations The first external evidence of brain development appears as a blastodermic thickening mid-dorsally in the cephalic region of 21-hour-old embryos In embryos 27 hours old there comes into prominence, dorsal to the prospective brain, a double mass of granular substance, representing the material for the development of the eye In adult individuals the eye is a single organ placed in front of the head but embryologically this structure has a double origin Other details in the course of the development of the embryos *in vitro* were also followed Embryos reared on depression slides when transferred to bottles containing the standard amount of the culture medium (Banta<sup>4</sup>) become sexually mature and produce young

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<sup>4</sup>Banta, A. M., *Physiology, Genetics and Evolution of Cladocera*, Carnegie Institution Publication No 513, 1939

# Changes in Structural Components of Human Body from Six Lunar Months to Maturity

HARRY A WILMER (Introduced by R E Scammon)

From the Department of Anatomy and the Graduate School, University of Minnesota

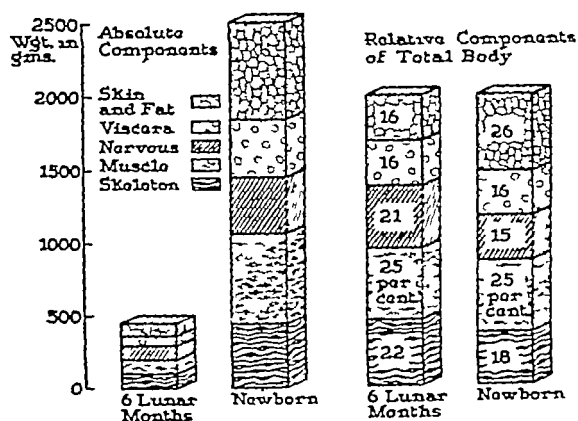
The changes of the various structural components of the human body in development are well illustrated by volumetric histograms. These are shown in Figs 1 and 2. They are based upon data collected in this laboratory as well as a series of records collated from the literature.

In this presentation the structural components of the body considered are skin and superficial fat (*panculus adiposus*), the visceral mass (including the heart), the nervous tissues (of both the peripheral and the central nervous systems), the voluntary musculature and the fresh ligamentous skeleton.

The periods represented are 6 lunar or fetal months, birth, and full maturity. Both sexes are included in each of the periods.

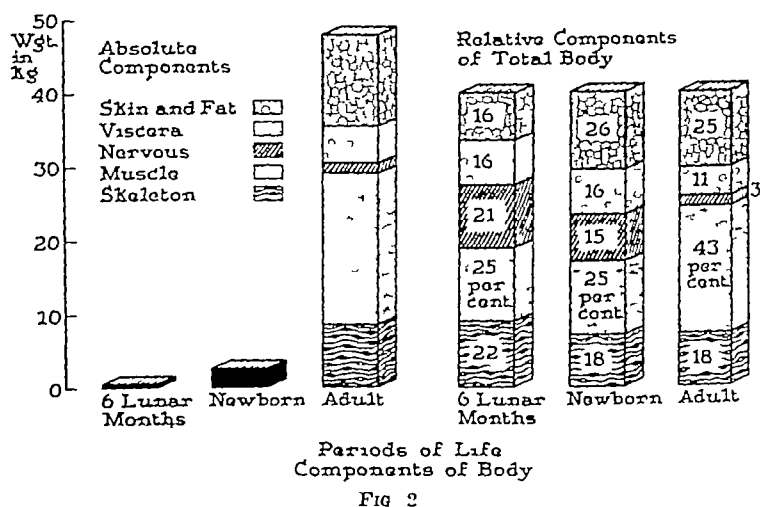
Figure 1 illustrates the differences in composition of the body at 6 lunar months and at birth. During this interval the net weight of the body increases approximately five-fold from 500 to 2500 g.

The relative changes in the distribution of the body components are: the skin and superficial fat increase from about one-sixth to over one-fourth of the body mass. The nervous tissue component and the



Prenatal Growth

FIG 1



skeletal component both show a marked relative decrease. The visceral and the voluntary muscle components remain almost constant.

Fig 2 shows the changes in components between birth and maturity. During this interval the net body weight increases almost twenty-fold. The postnatal changes in the relative distribution of components consist of a marked increase in the voluntary muscle from about one-fourth to over two-fifths of the body mass. But the visceral component decreases from about one-sixth to about one-tenth of the body mass, and the nervous tissue component from about one-seventh to about one-thirtieth. The skin and superficial fat component and the component of the skeleton remain practically unchanged.

TABLE I  
Absolute and Relative Weights of the Various Components of the Body for Both Sexes (Collated from Various Sources)

Body components	Absolute wt (g)			Relative wt (%)		
	6 lunar months	Newborn	Adult	6 lunar months	Newborn	Adult
Skin and superficial fat	73.5	666.35	12,395.5	16.21	26.43	25.61
Muscle	111.5	625.25	20,846.3	24.60	24.80	43.07
Skeleton	101.0	446.00	8,518.6	22.28	17.69	17.60
Total viscera	71.1	398.20	5,185.5	15.68	15.79	10.71
Central nervous system	96.2	385.50	1,455.0	21.22	15.29	3.01
Sum*	453.3	2,521.30	48,401.8	100.00	100.00	100.00

\* These sums do not include the weight of the blood or the weight lost in the determination of approximately 33 g in the 6 months fetus, 116 g in the newborn, 5270 g in the adult male and 4000 g in the adult female.

The numerical values for the absolute and relative sizes of all these components are shown in Table I

The most striking of this series of changes is the increase of the range of variability of the several components forming the body. At 6 lunar months there is a maximum difference of only 9% in their relative distribution. By birth there is a maximum difference of 40%. And at maturity there is a maximum difference of over 1000%. These computations are in terms of the smallest component in each series but they may also be demonstrated in terms of the largest component or in terms of absolute values.

11261

### Keto-Reacting Substances in the Bile of Dogs

A. L. BERMAN, E. SNAPP, V. S. HOUGH AND A. C. ILL

*From the Department of Physiology and Pharmacology, Northwestern University Medical School, Chicago*

A study of the literature revealed that certain investigators have identified and isolated various ketonic acids from the bile of different animals. Fernholz<sup>1</sup> isolated 3-hydroxy-6-ketocholanic acid from hog bile and studied its properties. Schoenheimer and others<sup>2, 3</sup> confirmed the isolation of this ketonic acid and further stated that the ketonic acids of hog bile comprise approximately 10% of the total crude acids. Wieland and Kishi<sup>4</sup> isolated and identified 3-hydroxy-12-ketocholanic acid from ox bile. Sobotka found 3-hydroxy-12-ketocholanic acid in human bile.<sup>5</sup>

No one apparently has reported a method for the quantitative determination of carbonyl or keto-reacting substances in bile. Since our work required such a method we have been fortunate at having at our disposal a quantitative method which had been developed by Dr. Gustus of the Wilson Laboratories, Chicago. The principle of this method is as follows: The carbonyl or keto groups in the bile are allowed to react with an excess of hydroxylamine. The remaining hydroxylamine is combined with diacetyl-monoxime, converting the

<sup>1</sup> Fernholz, E. *II S Z f Physiol Chem* 1935 **232**, 202.

<sup>2</sup> Schoenheimer, R., and Johnston, C. G. *J Biol Chem* 1937, **120**, 499.

<sup>3</sup> Ansel, M., and Schoenheimer, R. *J Biol Chem* 1938 **124**, 609.

<sup>4</sup> Wieland, H., and Kishi, S. *II S Z f Physiol Chem* 1933 **214**, 47.

<sup>5</sup> Sobotka, H. *Chem. Pct* 1934 **17**, 334.

latter to the dioxime compound. The dioxime is precipitated with nickel acetate, and the resulting precipitate washed, dried and weighed. By calculation, the amount of carbonyl groups that was originally present in the bile and that combined with the hydroxylamine can be obtained. The exact details of this analysis for carbonyl groups in bile will be published later by Dr. Gustus.

The accuracy of the method has been examined in two ways. First, the chemist was supplied with duplicate unknowns of the same bile obtained from 11 bile fistula dogs (Rous-McMaster method), some of which received no bile salts and others bile salts containing varying amounts of ketocholic acid. The analyses of the duplicate unknowns checked within  $\pm 6\%$  of the mean, except in one case in which the error was  $\pm 15\%$ . Second, pure triketocholic acid added to dogs' bile was recovered in amounts varying from 95% to 99%.

*Control output of keto-reacting substances.* Before ascertaining the effect of feeding natural and oxidized bile acids on the keto-reacting substances in bile, it was necessary to determine the amount of keto-reacting substances present in 'control' bile. By "control" bile is meant the bile that is secreted when the animal is fed the standard diet only and no bile or bile salts are administered. This was done in 55 tests on 15 dogs. The average control daily output of keto-reacting substances (Table I) amounted to  $252 \pm 16$  mg (S.E.M.) expressed as triketocholic acid or  $52.6 \pm 4.4$  mg of carbonyl groups ( $252 \times 209 = 52.6$ ). The daily keto output was subject to considerable individual variation under basal conditions, so that the output of keto-reacting substances ranged from 98 to 507 mg per 24 hours in the different dogs. One of the dogs showed a 50% variation between different control periods; the others varied only from 5 to 15%.

*Output of keto-reacting substances when natural or ox-bile salts were administered.* When natural ox-bile salts were administered, either in 3 or 5 g daily doses, there was an increase in keto groups secreted in the bile, i.e., 72.1 mg and 92.8 mg respectively as compared to 52.6 mg for "control" bile (Table I). The source of the increased keto-reacting substances in bile is problematic. However, as has been shown before and confirmed in this work, when unoxidized cholates are given about 10% are "lost" during one enterohepatic circuit. This loss of 10% is approximately balanced by the increase of 20 mg of keto groups (Table I). This is true only when it is assumed that the natural conjugated bile acids "lost" are changed to oxidized conjugated bile acids which contain 11.2% keto groups. This is reasonable because if the natural conjugated bile acids in cattle bile are oxidized they contain 11.2% keto groups.

TABLE I.  
Effect of Unoxidized and Oxidized Bile Acids on Content of Keto reacting Substances in Dog's Bile

Procedure	No of dogs	No of tests	Bile, cc/24 hr	Total O = O groups, mg/24 hr	Increase		Recov of keto acid fed	% recov
					O = O groups over control			
Control Diet without bile salts	15	55	126	52.6				
1.5 g cholic acid as cattle bile*	9	34	190	72.1		19.5		
1.0 g oxidized conjugated bile salts as they occur in cattle bile	11	14	175	107.2		54.6†	488†	16§
1.0 g keto-biolic acid from cattle bile	7	10	251	186.2		133.6	730	24
1.0 g dihydrocholic acid	6	10	204	227.3		174.7	836	28

\*Contains no keto reacting substances

†107.2 — 52.6 = 54.6

‡54.6 — 11.2 × 100 = 488

§133.6 — 18.3 × 100 = 730

§488 — 3000 = 16% Note that the per cent recovered is about proportional to the keto acid content of the oxidized bile acids fed

*Output of keto-reacting substances when oxidized or ketocholanic acids were administered* We used a product containing oxidized conjugated bile acids made from cattle bile (Dechacid No. 14, Wilson Laboratories, Chicago). That is, cattle bile was oxidized without splitting-off the taurine and glycine. This product contained by analysis 11.2% keto or carbonyl groups. We used another product containing oxidized unconjugated bile acids made from cattle bile (Ketochol, Searle, Chicago). This product contained by analysis 18.3% keto or carbonyl groups. We also used pure dehydrocholic acid (Decholin, Riedel-de Haen, New York), which contained by analysis 20.9% carbonyl groups.

When the oxidized bile acids were fed a marked increase in the keto-reacting substances in the bile of the dogs resulted (Table I). By subtracting the control output of keto-reacting substances from the total output, one may obtain the increase due to the keto-acids fed. This increase may be assumed to be the amount of keto-acid fed that is recovered in the bile. In this way the recovery of the keto acid fed may be estimated. Though two other assumptions are possible, we believe the one mentioned is the most logical, since the increase in keto-reacting substances in bile after unoxidized bile acids are fed appears to be due to oxidation of the bile acid lost during an enterohepatic circuit rather than to choleresis *per se*.

*Conclusions* (1) Keto-reacting substances are present in dogs' bile and can be quantitatively determined. (2) The output of keto-reacting substances is increased slightly when unoxidized bile acids are fed. (3) When oxidized bile acids or ketocholanic acids are administered orally, the output of keto-reacting substances in the bile is markedly increased. By assuming that the increase over the control output is due to the ketocholanic acid fed, one may calculate the recovery in the bile of the ketocholanic acid administered.

## Production of Neutropenia in Swiss Mice by Injection of Potassium Dicarboxy-benzanthracene

HAZEL A C LIN\* AND RAPHAEL ISAACS

*From the Thomas Henry Simpson Memorial Institute for Medical Research, and the Department of Gynecology, University of Michigan*

Leukopenia, found in one of Dr A T Bradbury's Swiss mice dying after the injection of dicarboxy-benzanthracene, led to the investigation of the blood changes produced by this drug

Swiss mice, both male and female, were used, the 9 controls being kept in the same cages and on the same food as the injected animals. Blood was taken from the tail, using the standard technic, and United States Bureau of Standards checked apparatus. The 9 experimental animals received subcutaneous injections of 5 mg of water-soluble potassium salt of dicarboxy-benzanthracene on 2 occasions one week apart. The mice and corresponding controls varied in age from 1½ to 3½ months.

In normal Swiss mice untreated the average of 36 red blood cell counts on 18 mice was  $8,930,000 \pm 1,150,000$  per cu mm. The average of 86 white blood cell counts on 18 normal Swiss mice was  $10,100 \pm 3,600$  per cu mm. The absolute number of polymorphonuclear neutrophils (average of 94 counts on 18 untreated animals) was  $5,848 \pm 2,713$  per cu mm.

One week after a single injection of the soluble potassium salt of dicarboxy-benzanthracene the average total white blood cell count fell to  $8,655 \pm 4,000$  per cu mm and in 2 weeks the average fell to  $7,300 \pm 2,600$  per cu mm. The decrease was primarily the result of a fall in the number of neutrophils, as shown in Table I.

The eosinophils averaged 153.2 per cu mm in 93 counts in non-treated mice, with extremes of 0 to 510 per cu mm. The variation after injection of the benzanthrane solution was not significant.

TABLE I

Control period—Absolute No. of neutrophils	$5,448 \pm 2,713$ per mm <sup>3</sup>
One week after first injection	$4,569 \pm 2,100$ " "
Second week after first injection	$2,623 \pm 1,070$
Received second injection at this time	
Third week after first injection	$1,209 \pm 627$
Fifth " " " "	$1,650 \pm 890$
Sixth " " " "	$3,613 \pm 1,585$
Seventh " " " "	$4,437 \pm 2,215$
Eighth " " " "	$5,518 \pm 957$

\* Peiping Union Medical College, Peiping, China



Before treatment, the average number of monocytes was 7024 per cu mm (average 6.8%, extremes 1-11%). The variations 1, 2, 3, 5, 6, 7, and 8 weeks after the first injection were (averages) 672, 588, 510, 417, 678, 652, and 903 per cu mm

Before treatment the lymphocytes averaged 3,735 per cu mm (36.16%, extremes, 15 and 62%). The variations 1, 2, 3, 5, 6, 7, and 8 weeks after the first injection were (averages) 3241, 4281, 4283, 4201, 4953, 3829, 3189 per cu mm. Thus, while the lymphocytes were as high as 70 to 80% during the post-injection period, the absolute numbers did not vary greatly, the maximum increase (average) being 1218 per cu mm

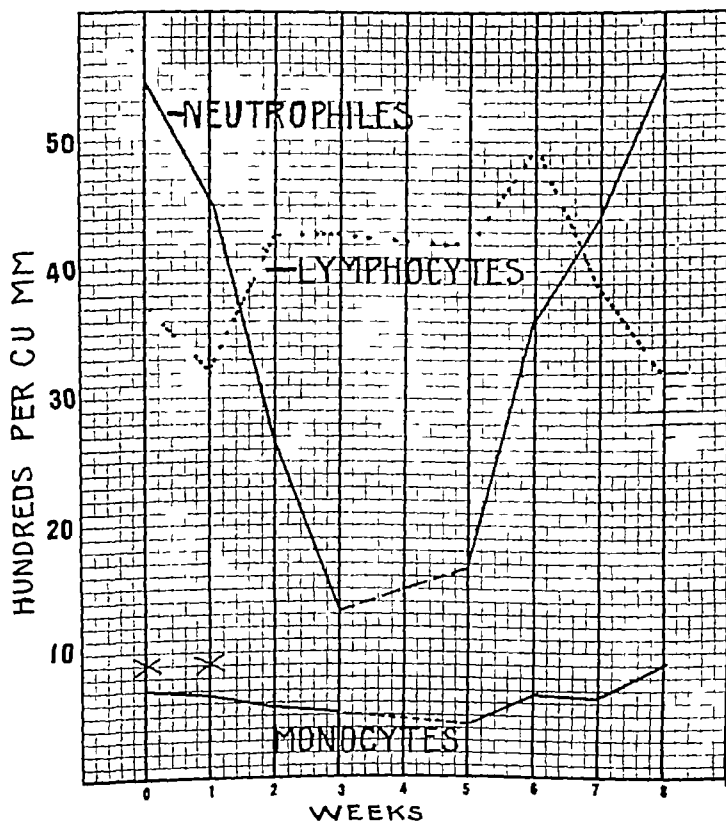


Fig 1

The leucocyte changes after injection of dicarboxy benzanthraccene. The curves represent the averages for 9 mice.

The point marked "O" represents the average for a control period of 2 weeks. Injections were made at the points marked "X".

There was no significant change in the number of basophils, which varied from 0 to 1%

*Summary and Conclusions* After the injection of the water-soluble potassium salt of dicarboxy-benzanthracene into Swiss mice there is a marked decrease in the number of polymorphonuclear neutrophils a slower, and moderate decrease in the number of monocytes, with but slight changes in the number of lymphocytes, eosinophils, and basophils. Recovery is complete 7 weeks after the last injection. Data on the blood of normal Swiss mice are given.

## 11263

# Relation Between Growth of Pneumococcus III and Concentration of Capsular Polysaccharide Appearing in Culture Filtrates \*†

SAMUEL CHARLES BUKANTZ,† (Introduced by Jesse G. M. Bullowa)

*From the Littauer Pneumonia Research Fund, New York University College of Medicine, and the Medical Service, Harlem Hospital, Department of Hospitals, New York City*

A method of adapting the photoreflexometer<sup>1</sup> to the quantitative determination of pneumococcal capsular polysaccharide in solutions of unknown concentration has been described<sup>2</sup>. In the present investigation, the method was employed to determine the concentrations of SSS III appearing in Seitz filtrates of blood broth cultures following inoculation with varying amounts of a standardized strain of *Pneumococcus* III; these concentrations were correlated with the phase and amount of growth of the organisms.

A strain of *Pneumococcus* III obtained from the sputum of a pneumonia patient was brought to constant virulence after the suggestions of Schmidt and Hilles.<sup>3</sup>  $5 \times 10^{-4}$  ml of a 12-hour blood-

\* These studies received financial support from the Metropolitan Life Insurance Company, and from Mr. Bernard M. Baruch, Mr. Bernard M. Baruch, Jr., Miss Belle N. Baruch, and Mrs. H. Robert Samstag.

† Littauer Fellow in Pneumonia Research.

‡ Technical assistance in this work was rendered by Miss Anita Cooper.

<sup>1</sup> Libby, R. L., *J. Immunol.*, 1938, **34**, 71, 268.

<sup>2</sup> Bukantz, S. C., and Bullowa, J. G. M., *Proc. Soc. Exp. Biol. and Med.*, 1940, **43**, 418.

<sup>3</sup> Schmidt, L. H., and Hilles, C., *J. Inf. Dis.*, 1939, **65**, 273.

Before treatment, the average number of monocytes was 7024 per cu mm (average 6.8%, extremes 1-11%). The variations 1, 2, 3, 5, 6, 7, and 8 weeks after the first injection were (averages) 672, 588, 510, 417, 678, 652, and 903 per cu mm.

Before treatment the lymphocytes averaged 3,735 per cu mm (36.16%, extremes, 15 and 62%). The variations 1, 2, 3, 5, 6, 7, and 8 weeks after the first injection were (averages) 3241, 4281, 4283, 4201, 4953, 3829, 3189 per cu mm. Thus, while the lymphocytes were as high as 70 to 80% during the post-injection period, the absolute numbers did not vary greatly, the maximum increase (average) being 1218 per cu mm.

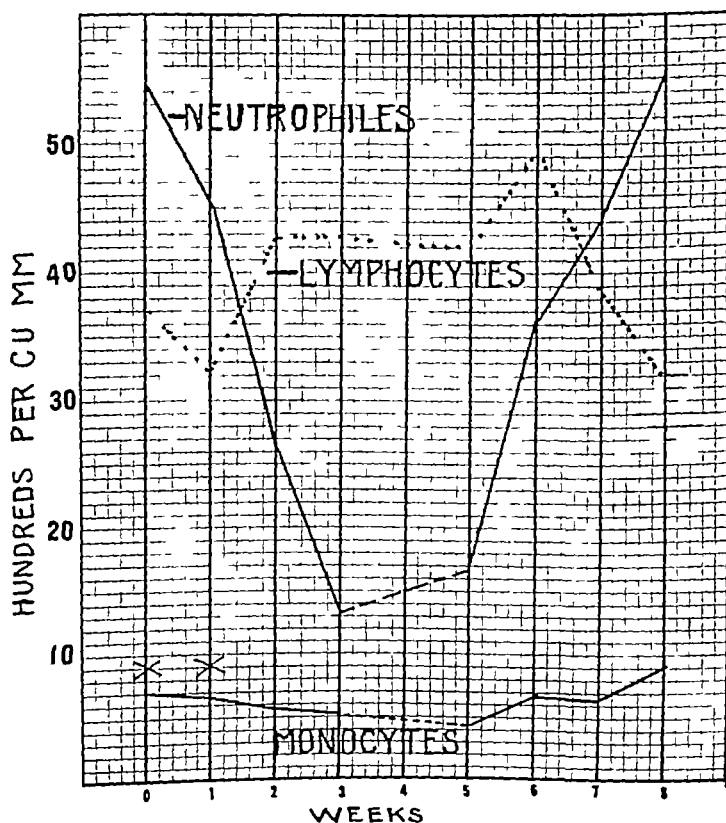


FIG 1

The leukocyte changes after injection of dicarboxy benzanthracycline. The curves represent the averages for 9 mice.

The point marked "O" represents the average for a control period of 2 weeks. Injections were made at the points marked "X."

TABLE I  
 Titration of Various Filtrates from Experiment II (9,000 Organisms per ml Inoculum) with Standard Serum III Sensitivity of  
 Photoreflexometer at 15

Hour		Dilution of Filtrate in Broth											Avg SSS per ml
		Und	1.5	1.10	1.20	1.30	1.35	1.40	1.50	1.60	1.80	1.100	
10	Corrected Photon Rdg <sup>1</sup> SSS Equiv of Dilution <sup>2</sup> SSS Conc in Orig Fil <sup>3</sup>		5.0		0.0075 0.15	0.004 0.12		1.5 Ind	1.0 Ind				0.13
12 and 50'	C P R SSS Eq Dil <sup>2</sup> SSS Or Fil <sup>3</sup>				8.5 0.0125 0.37			5.0 0.0075 0.37		3.0 0.004 0.32	2.0		0.35
24	C P R SSS Eq Dil <sup>2</sup> SSS Or Fil <sup>3</sup>					20.0 0.032 13		16.0 0.025 13		8.0 0.012 10	7.0 0.01 10		12
28	C P R SSS Eq Dil <sup>2</sup> SSS Or Fil <sup>3</sup>		17.5 E A <sup>4</sup>	24.0 E A		21.0 0.033 13		15.5 0.023 14		12.5 0.019 15	8.0 0.012 12		13
32	C P R SSS Eq Dil <sup>2</sup> SSS Or Fil <sup>3</sup>		14.0 F A <sup>4</sup>	24.0 F A		20.5 0.033 13		13.0 0.0195 12		10.0 0.015 12	6.5 0.01 10		12
48	C P R SSS Eq Dil <sup>2</sup> SSS Or Fil <sup>3</sup>		7.5 F A <sup>4</sup>	19.0 E A	23.0 E A	23.5 23.5		23.0 0.034 17		15.0 0.022 18	12.0 0.018 18	8.0 0.012 18	18

<sup>1</sup> Corrected Photoreflexometer Readings—Obtained by subtracting the dry blank reading of the cell from the final turbidity reading

<sup>2</sup> SSS (Capsular Polysaccharide) Equivalent of Dilution—Obtained by reference to a previously determined standard curve. This figure expresses the actual amount of SSS present in the 1 ml sample of the dilution titrated

<sup>3</sup> SSS Concentration in the Original Filtrate—Obtained by multiplying SSS Equiv of Dil by the Dilution [i.e., For 10 hr 0.0075  $\times$  20 = 0.15] expressed as milligrams per milliliter

<sup>4</sup> E A—Excess of antigen

<sup>5</sup> Ind—Indeterminate, because of inaccuracy in calculations of points on lowermost extrapolations of the standard curve

broth culture of the strain was injected intraperitoneally into an 18 g mouse. Twelve hours after the inoculation the mouse was sacrificed and a standard loop of heart's blood of the freshly killed animal was inoculated into 10 ml of blood broth. This cycle was repeated daily and used routinely for the preservation of the strain. After the eleventh and twelfth passages, the M L D of the strain was found to be the same—8 organisms. Subsequent determinations have indicated that the virulence has not changed, even after the 50th passage. The 12-hour cultures contain a fairly constant number of organisms, usually between  $50 \times 10^6$  and  $100 \times 10^6$  per ml.

In the following experiments, all bacterial counts were made by the plating method with dilutions in broth. Separate pipettes were used for each dilution which was mixed 25 times before transfer. One ml of diluted culture, containing the desired inoculum, was seeded into each of a series of tubes containing 9 ml of 4% horse-blood-broth. A colony count of the original culture was performed, at the same time, in order to determine the size of inoculum used. All inoculated tubes were then incubated at  $37^\circ\text{C}$  for the duration of the experiment. At 2, 4, 6, 8, 10, 12, 24, 28, 32, and 48-hour intervals, one tube was removed, a colony count performed and the contents of the tube filtered through the Swinney Seitz Filter Adapter. All filtrates were sterile, and the pH, determined with nitrazine paper, was at least 7.0, even after a 48-hour period of incubation. The concentration of capsular polysaccharide in each filtrate was then determined by titration of 1 ml of varying dilutions of each filtrate with 1 ml of a standardized serum in the zone of antibody excess, photorefractometric measurements were made after incubation of each mixture in refraction cells for 20 minutes at  $37^\circ\text{C}$ . Dilutions of the filtrates were made in broth and the concentrations determined by reference to a standard curve prepared by titrating varying dilutions of the polysaccharide in broth, with the standard serum. Filtration control studies indicated that filtration of SSS in broth was not quantitative until adsorption by the filter had been completed. This adsorption occurred most rapidly in the presence of large numbers of bacteria ( $100,000,000$  per ml) and the higher concentrations of SSS.

§ According to the modification of the Park Cooper technique employed by The Massachusetts Antitoxin and Vaccine Laboratory (White, *Biology of the Pneumococcus*, page 653).

|| In preliminary experiments 4 tubes separately inoculated with the same number of pneumococci grew out an identical number of organisms in the same time.

¶ Beeton Dickinson, manufacturers, Rutherford, N. J.

\*\* The original broth contained no glucose.

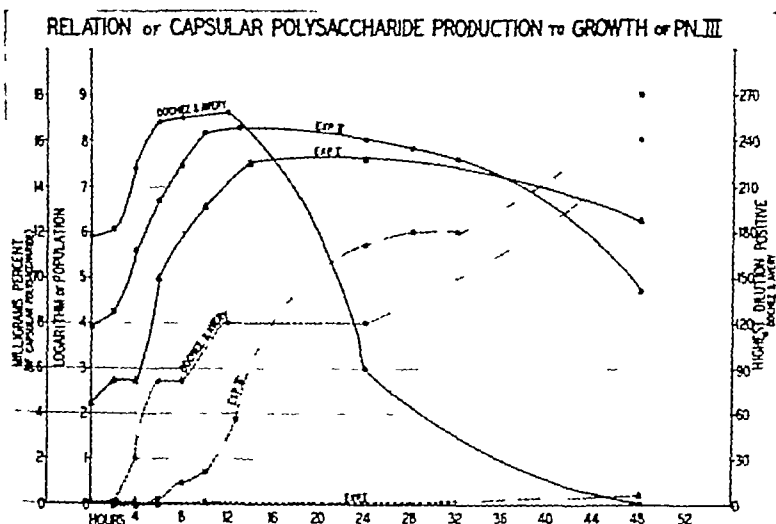


FIG 1

The relationship of increases in concentration of capsular polysaccharide III in culture filtrates to the phase of growth of *Pneumococcus* III in blood broth, two different sizes of inoculation of an organism having the same virulence in each experiment.

Solid lines ————— Logarithm of populations  
Broken lines ————— Experiments I and II, Concentration of SSS in mg per 100 ml (mg%)

Broken lines ————— Dochez and Avery, highest dilution of SSS giving a positive reaction with the solution of antibody used by them.

Experiment I—Inoculum of 165 organisms per ml

Experiment II—Inoculum of 9,000 organisms per ml

growth-rate of the organisms In Fig 1 the logarithms of colony counts and mg % of polysaccharide produced are plotted as ordinates, with time as abscissa

Dochez and Avery<sup>4</sup> concluded, from their studies, that the largest quantity of capsular polysaccharide was found in those pneumococcal culture filtrates obtained during the phase of maximal growth of the organism. The present observations indicate that the greatest quantity of capsular polysaccharide is found in filtrates of blood-broth cultures obtained after the maximal growth phase is completed, i.e., after the completion of the logarithmic phase of growth. In this regard it was interesting to plot the logarithms of the population and the polysaccharide production described in Dochez and Avery's protocol (Polysaccharide concentration was plotted as the highest dilution giving a positive reaction, since a dilution method was em-

<sup>4</sup> Dochez, A. R., and Avery, O. T., (a) *PROC. SOC. EXP. BIOL. AND MED.*, 1917, 14, 126, (b) *J. Exp. Med.*, 1917, 26, 477

(10-20 mg %) However, following a very large initial inoculum (900,000 per ml, Experiment III), low concentrations of SSS were obtained in early filtrates despite the presence of large numbers of bacteria (63,000,000 per ml), while later filtrates, obtained from cultures having approximately the same number of bacteria, yielded considerably higher concentrations of SSS. The influence of autolysis upon the increase of concentration of SSS has not yet been adequately investigated because of the difficulty in defining a technic for quantitative determination of the degree of autolysis. Judging from the rate of decrease of viable organisms, however, there is at least one period of significant increase in concentration of SSS in filtrates which appears to be unrelated to autolysis (13th to 24th hour, Experiment II) and more probably represents an alteration in the physiologic activity of the organisms.

A small quantity of capsular polysaccharide (0.5 mg %) was found after 48 hours following an initial inoculum of 165 organisms per ml (Experiment I). A much larger quantity of polysaccharide (18 mg %) was found after an initial inoculum of 9,000 organisms per ml (Experiment II). Table I summarizes the titrations of some of the filtrates obtained following the inoculation of 9,000 organisms per ml. The remaining filtrates were titrated in the same way. Table II summarizes the results of this experiment in relation to the

TABLE II

Experiment II. Growth of highly virulent pneumococcus III in 4% horse blood broth following an initial inoculum of 9,000 organisms per ml. Concentrations of SSS determined with the photon reflectometer.

Hrs.	Colony count	Log of count	*No of generations	†Generation time (min)	SSS concentration mg/100 ml
2	18,200	4.26	1.0	120	0
4'30"	384,500	5.59	4.4	34	0
6'40"	5,100,000	6.71	3.7	35	18
8	24,060,000	7.38	2.2	36	70
10	138,000,000	8.14	2.5	48	130
12'50"	170,000,000	8.23	0.3	566	35
24	105,000,000	8.02	—	—	120
28	75,000,000	7.88	—	—	130
32	47,500,000	7.68	—	—	120
48	40,000	4.60	—	—	180

\* From formula for law of geometric progression

Log of Final Count — Log of Initial Count

$$N = \frac{\text{Log of Final Count} - \text{Log of Initial Count}}{\text{Log 2}}$$

† Time in minutes

$$\dagger = \frac{\text{Time in minutes}}{\text{Number of generations}}$$

} After Chesnev<sup>7</sup>

<sup>7</sup> Chesnev, A. M., *J. Exp. Med.*, 1916, **24**, 387

extracts Furthermore, we succeeded in producing total regressions in spontaneous breast carcinomas in 27 mice by intravenous injections of spleen extracts

This report deals with the action of another extract on spontaneous malignant tumors in mice, namely a yeast extract

Nevorojkin<sup>3</sup> injected concentrated suspensions of yeast into transplanted tumors and noticed retardation of growth and in some cases regressions

Maisin, Pourbaix and Caeymaex<sup>4</sup> demonstrated that they could retard the growth of experimental cancer in rabbits by adding boiled yeast to the food of the animals

The yeast extract which was used by us for intravenous or subcutaneous therapy of malignant tumors was prepared in the following manner 2500 g of Brewer's yeast is washed several times with distilled water The yeast is boiled for 7 minutes with 9000 cc distilled water to which 0.9 cc of glacial acetic acid has been added The extract is filtered through paper-pulp and concentrated *in vacuo* at 70°C to 1500 cc It is precipitated with 1500 cc of absolute alcohol The filtrate is concentrated *in vacuo* at 70°C to 900 cc

The yeast extract was used intravenously sometimes in combination with subcutaneous injections The average dose for intravenous treatment was 0.1 cc, whereas 0.5 cc was injected subcutaneously The intravenous injections were given into the tail of the mouse When used subcutaneously the extract was injected as far away from the site of the tumor as possible in order to avoid the possibility of direct action of the extract on the tumor Daily, or on alternate days, injections were given until the tumor disappeared or were discontinued when the tumor did not respond to treatment Injections of yeast extract were continued after disappearance of the tumor in order to avoid a possible recurrence

Thirty-three tumor-bearing mice were used for these experiments Twenty-two came from the Jackson Memorial Laboratory Strain A Eleven were bought from the Rockland Farm They did not belong to a pure inbred strain

The average size of the tumor at the onset of the treatment can be recognized from the sketches of the regressed tumors in Table I

In each instance the malignant character of the tumor was established by biopsy before treatment was started The histologic criteria of malignancy were chiefly cytologic such as many atypical mitoses

<sup>3</sup> Nevorojkin, J., *Festnik Roentgenol.*, 1935, **15**, 344

<sup>4</sup> Maisin, J., Pourbaix, Y., and Caeymaex, P., *Comptes rendus Soc. biol. Paris* 1938, **127**, 1477



ployed by them in estimating polysaccharide produced ) These are also represented in Figure I, it is apparent that the major portion of polysaccharide in their experiment was found after completion of the logarithmic phase of growth Of additional interest are the recent observations of Hoogerheide,<sup>1</sup> that the size of the capsule surrounding *Klebsiella pneumoniae* (*Bact Friedlander*) begins to increase only after the completion of the logarithmic phase of growth, and the earlier observations of Cowan<sup>2</sup> that hemolysin appears in pneumococcal broth cultures only after completion of the logarithmic phase of growth

*Summary* The concentration of capsular polysaccharide appearing in blood-broth culture filtrates following the growth of a highly virulent, standardized strain of *Pneumococcus III*, was determined photomicroreflectometrically and correlated with the growth rate of the organism An initial inoculum of 165 organisms per ml yielded a very small quantity of polysaccharide (0.5 mg %) An inoculum of 9,000 organisms per ml yielded 18 mg % of capsular polysaccharide in 48 hours The greatest increases in concentration of polysaccharide occurred in two stages after the completion of the logarithmic phase of growth

## 11264

### Effect of Intravenous Injections of Yeast Extract on Spontaneous Breast Adenocarcinomas in Mice

R. LEWISOHN, C. LEUCHTENBERGER, R. LEUCHTENBERGER AND D. LASZLO (Introduced by Gregory Schwartzman)

*From the Laboratories of the Mount Sinai Hospital, New York City*

In this laboratory, studies on the action of different spleen extracts on transplanted and on spontaneous tumors have been carried out for several years It was shown in previous publications<sup>1, 2</sup> that the complete retrogression of transplanted Sarcoma 180 could be accomplished in 60% of the treated animals following subcutaneous injections of biologically<sup>2</sup> or chemically concentrated spleen

<sup>1</sup> Hoogerheide, J. C., *J. Bact.*, 1939, **38**, 367

<sup>2</sup> Cowan, S. T., *J. Path. and Bact.*, 1934, **38**, 61

<sup>1</sup> Lewisohn, R., *Surg., Gyn. and Obst.*, 1938, **66**, 563

<sup>2</sup> Lewisohn, R., Leuchtenberger, R., and Laszlo, D., Internat. Cancer Congress, Atlantic City, Sept., 1939

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The average size of the tumor at the onset of the treatment can be recognized from the sketches of the regressed tumors in Table I

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<sup>3</sup> Nevorojkin, J, *Vestnik Roentgenol.*, 1935, 15, 344

<sup>4</sup> Maisin, J., Pourbaix, Y., and Caeymaex, P., *Comptes rendus Soc. biol. Paris* 1938, 127, 1477

Table I Effect of yeast extract on spontaneous breast adenocarcinomas in mice JML-Jackson Memorial Laboratory R.F.-Rockland Farms

No	Source	Actual size of tumor	Treatment started	Healed	Injections	
					Intravenous	Subcutaneous
310	JML		Nov 9 1939	Nov 25 1939	11	0
350	"		10,	Dec. 20 "	13	11
352	"		" 10 "	Jan 9 1940	13	14
362	"		" 10 "	Dec. 16 1939	11	8
402	"	 2 separate tumors 1 anterior 1 lateral	Dec 23	Jan 13 1940	11	10
404	"	 Hemorrhagic	" 23 "	16	12	10
R 18	R F		Jan. 13 "	" 22 "	7	7
R 22	"		" 15 "	21, "	5	4

and striking cell irregularities, because actual invasion of the adjacent healthy tissue could hardly be established in a biopsy which had to be made as small as possible

Controls were first deemed unnecessary since it has generally been assumed and has been confirmed by personal communication from Dr Little that breast carcinoma of Strain A Jackson Memorial Laboratory, proven by biopsy, never disappears spontaneously. However, we believed it was necessary to establish whether biopsy alone might not influence the natural evolution of this tumor. In 60 mice, Strain A Jackson Memorial Laboratory, and in 5 Rockland Farm mice, in which only biopsy was performed, we have not observed so far disappearance of the tumor (Strain A Jackson Memorial Laboratory). However, temporary regressions in size were noted.

Of 33 animals treated with yeast extract, the tumors disappeared in 8 mice and have not recurred so far (see table). Naturally, the animals remain under further observation. In 10 animals the tumor was reduced in size. In 15 instances either no change in the size of the tumor was noted or they had even increased in size when treatment was discontinued or the animals died.

Details of the treatment and the source of the 8 mice in which the tumors regressed following treatment with yeast extract are presented in Table I.

It is impossible in this brief presentation to describe in detail the

interesting microscopic changes which occur in tumors during the treatment with yeast extract. Extensive cell necrosis presents a very striking picture which will be described in a subsequent publication.

Furthermore, we postpone for a later date reports on the treatment of malignant tumors with yeast in powder form or in pellets.

The factors responsible for this action of yeast extract are of course entirely unclear. Certainly, many different possibilities must be considered. For instance, it remains to be determined whether bacterial factors which may have been present in yeast extracts were operative in the effect described. Duran-Reynals<sup>5</sup> observed total regressions of some spontaneous mammary carcinomas of mice due to repeated injections of bacterial filtrates.

*Summary* In 8 out of 33 treated mice complete regressions of spontaneous breast adenocarcinomas were effected with intravenous and subcutaneous injections of yeast extract.

## 11265 P

### Action of Sulfathiazole and Sulfamethylthiazole on *Staphylococcus aureus*

GEOFFREY RAKE AND C M MCKEE

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Studies<sup>1-4</sup> on certain thiazole analogues of sulfapyridine<sup>5, 6</sup> have indicated that these drugs have a therapeutic activity equal to that of sulfapyridine when tested against pneumococci, streptococci, meningococci and the agent of lymphogranuloma venereum and that the toxicity of sulfathiazole itself is usually less than that of sulfapyridine. More recently there have appeared several communications dealing with the activity of sulfathiazole and certain of its derivatives

<sup>5</sup> Duran Reynals, F, *Proc Soc Exp Biol and Med*, 1935, **32**, 1517

<sup>1</sup> van Dyke, H B, Greep, R O, Rake, Geoffrey and McKee C M, *Proc Soc Exp Biol and Med*, 1939, **42**, 410

<sup>2</sup> McKee C M, Rake Geoffrey, Greep, R O and van Dyke, H B, *Proc Soc Exp Biol and Med*, 1939, **42**, 417

<sup>3</sup> Rake Geoffrey, van Dyke H B, Corwin, W C, McKee, C M, and Greep, R O, *J Bact*, 1940, **30**, 45

<sup>4</sup> Cooper, F B, Gross, Paul, and Lewis, Marion, *Proc Soc Exp Biol and Med*, 1939, **42**, 421

<sup>6</sup> Foshander R T and Walter L A, *J Am Chem Soc*, 1939, **61**, 2032

<sup>6</sup> Jott W A and Bergheim Frank H, *J Am Chem Soc*, 1939, **61**, 3593

on staphylococci.<sup>7-10</sup> Herrell and Brown<sup>7</sup> found much greater *in vitro* bacteriostatic activity of sulfamethylthiazole than of sulfathiazole against *Staphylococcus aureus*. However, they give only one experiment and that in brief. They also state that "much more marked protection" was accorded mice infected with *Staphylococcus aureus* when these animals were treated with sulfamethylthiazole than when treated with sulfathiazole, but give no details. Lawrence<sup>8</sup> found that sulfamethylthiazole had a greater bacteriostatic activity than had sulfathiazole but his results would seem to be in part vitiated by the use of supersaturated solutions. Barlow and Homburger,<sup>9</sup> using small numbers of mice, give results which indicate little if any greater therapeutic activity of sulfamethylthiazole as compared with sulfathiazole. Finally, Helmholtz<sup>10</sup> found that voided urine from patients treated with either of these two drugs was markedly bactericidal for *Staphylococcus aureus* even with drug concentrations as low as 16 mg %.

In carrying out *in vitro* studies with sulfathiazole, sulfamethylthiazole and sulfapyridine it has been found that minor variations in the technic used or in the broth menstruum have greatly influenced results. When, however, concentrations of drug of approximately 200 mg % are used and supersaturated solutions are avoided, it has been found that the methyl derivative has greater bacteriostatic activity than sulfathiazole which in turn, is more active than sulfapyridine. Table I shows such an experiment.

Poured plates inoculated with 0.1 ml of different dilutions of the

TABLE I  
Action of Sulfathiazole (ST), Sulfapyridine (SP), and Sulfamethylthiazole (SMT) on *Staphylococcus aureus* *in vitro*

	ST	SP	SMT	Control
Immediate	40*	40	80	20
2 hr	80	50	140	230
4 "	170	1170	140	7100
6 "	1800	9200	1300	530,000
24 "	460 million	1260 million	74 million	1580 million

Drug concentrations in each case 198 mg% added to broth after autoclaving

\*Figures indicate number of colonies per ml

Inoculum was 0.1 ml of 10<sup>-6</sup> dilution of 18 hr culture of strain MK in 2 ml volume.

<sup>7</sup> Herrell, W. E., and Brown, A. E., *Proc Staff Meetings Mayo Clinic*, 1939, **14**, 753

<sup>8</sup> Lawrence, C. A., (a) *J. Bact.*, 1940, **30**, 46, (b) *Proc Soc Exp Biol and Med*, 1940, **43**, 92

<sup>9</sup> Barlow, O. W., and Homburger, E., *Proc Soc Exp Biol and Med*, 1939, **42**, 792

<sup>10</sup> Helmholtz, H. F., *Proc Staff Meetings Mayo Clinic*, 1940, **15**, 65

cultures were made at the time intervals indicated. Counts were made and those recorded are from plates which gave the largest number of easily countable colonies.

Other experiments under similar conditions but with colony counts at different intervals of time confirm the relationship of activity, namely, sulfamethylthiazole > sulfathiazole > sulfapyridine. In certain cases, however, sulfapyridine and more particularly sulfamethylthiazole have not remained in solution during the test presumably because the starting solution was supersaturated. It would seem essential to avoid such precipitation of drug which may vitiate the results by decreasing the total amount of drug in solution. This condition of supersaturation seems to be rather a factor of the constitution of the broth than of the actual concentration of drug. Other factors, such as autoclaving the drug in the broth (with or without added glucose) or adding the drugs to the broth after the latter has been autoclaved (as in Table I) have also altered the results.

The agar cup plate method of testing *in vitro* activity<sup>11</sup> was also used\*. When the drugs were mixed with 5% gum acacia, sulfathiazole was found to be the most active compound producing inhibition of growth in a dilution of 1:8000 as compared to 1:3000 for sulfamethylthiazole and 1:1000 for sulfapyridine. Differences in solubility and diffusibility probably account for these differences in activity.

In the *in vivo* experiments drugs were administered to mice in 1% of their normal dry diet.<sup>2</sup> Five different strains of *Staphylococcus aureus*, 3 hemolytic and 2 non-hemolytic, were used. The mice were inoculated intraperitoneally and received in each case approximately 500,000 organisms suspended in 1 ml of 5% mucin. Mice were kept on the drugs for 10 days and then observed for an additional 21 days after return to normal diet. At the end of the 31-day period survivors were autopsied and the persistence of infection noted by obtaining positive cultures from one or all of the following organs: spleen, liver and kidney. Deaths after 7 days were very unusual.

As seen in Table II, sulfapyridine had very little if any activity. Both sulfathiazole and sulfamethylthiazole protected mice and the former drug was slightly but consistently better than the latter, as shown both by the number of surviving mice and by the percentage of residual infection in the organs as determined at autopsy. It may be noted that rough and other variants were found among the strains of

<sup>11</sup> Ruchle, C. L. A. and Brewer, C. M., U. S. Dept. of Agriculture, 1931, circular No. 198.

\* We wish to express our thanks to Dr. Brandt Rose of Philadelphia for suggesting the use of this test.

TABLE II.  
Action of Sulfathiazole, Sulfapyridine and Sulfamethylthiazole *in vivo*

Strain	No of mice in each group	ST	SP	SMT	Control
MK NH	30	22*	3	22	0
Co NH	40	17	4	14	3
469 H	30	15	2	9	2
687 H	30	21	3	16	1
631 H	20	20	6	18	2
Total	150	95	18	78	8
% of survivors whose organs gave positive cultures		14%	18%	25%	12.5%

H—Hemolytic strain NH—Non hemolytic strain

\* Figures indicate number of survivors over 31 day period

*Staphylococcus aureus* recovered from the mice treated with each of the 3 drugs

**Summary** In *in vitro* experiments with *Staphylococcus aureus* sulfamethylthiazole has shown greater bacteriostatic activity than sulfathiazole, the activity of the latter was in turn greater than that of sulfapyridine. In *in vivo* experiments with the same organism sulfapyridine has had little if any, activity. Both sulfathiazole and sulfamethylthiazole have protected mice and the former drug has been slightly but consistently more active than the latter.

## 11266

### Mechanism of the Blood Pressure Response to Anoxia During Hypoglycemia

A VAN HARREVELD AND W. L. MCRARY

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Gellhorn, Ingraham and Moldavsky<sup>1</sup> found that breathing a mixture of 6.2% oxygen in nitrogen caused a marked rise in blood pressure in dogs which had been made hypoglycemic by the injection of insulin. Intravenous injection of glucose made this blood pressure

<sup>1</sup> Gellhorn, E., Ingraham, R. C., and Moldavsky, L., *J. Neurophysiol.*, 1938, 1, 301

response disappear. Since, according to Ingraham and Gellhorn,<sup>2</sup> this rise in blood pressure is not due to a discharge of adrenalin, the authors first mentioned assumed that this phenomenon is caused by a "tremendous excitation of the sympathetic centers." Lambert and Gellhorn<sup>3</sup> reported (contrary to Heymans, Nowak and Samaa<sup>4</sup>) that the rise in blood pressure caused by the inhalation of a gas mixture low in oxygen is not due to a direct stimulation of the medullary centers but to a stimulation of the chemoreceptors in the carotid sinus and in the thorax, causing through a reflex the rise in blood pressure. Therefore Gellhorn, Ingraham and Moldavsky think it probable that the responsiveness of the medullary centers to these impulses is increased by hypoglycemia.\*

The same phenomena have been observed in man by Kraines and Gellhorn<sup>5</sup> under similar conditions, and Gellhorn<sup>6</sup> has used the above assumptions to explain the therapeutic effect of insulin shock in cases of schizophrenia.

When trying to explain the large rise in blood pressure from anoxia during hypoglycemia it should be kept in mind that the blood pressure is the resultant of a large number of often opposite reactions. Heymans and Bouckaert<sup>7</sup> emphasized that the excitability of the central nervous system (c.n.s.) is an important factor in determining the result of anoxia on the blood pressure.

To investigate the influence of the excitability of the c.n.s. on the blood pressure response to anoxia, this reaction was studied at various levels of narcosis. The blood pressure was recorded with a mercury manometer connected with one of the carotid arteries. The dye "fastusol BBA" served as an anticoagulant.<sup>8</sup> In very superficial narcosis the blood pressure response of dogs, cats and rabbits to breathing nitrogen or a gas mixture of 4.7% oxygen in nitrogen for 1 to 2 minutes (either spontaneously or by artificial respiration) was inconsistent: sometimes a small rise resulted but in other experiments an appreciable fall of the blood pressure was observed. In all experiments deepening of narcosis (chloralose or nembutal) increased

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- Ingraham, R. C. and Gellhorn, E., *Proc. Soc. Exp. Biol. and Med.*, 1939, **40**, 315.

<sup>2</sup> Lambert, E., and Gellhorn, E., *Proc. Soc. Exp. Biol. and Med.*, 1938, **38**, 427.

<sup>4</sup> Heymans, C., Nowak, S. J. G. and Samaa, A., *Compt. Rend. Soc. Biol.*, 1934, **117**, 248.

\* Assuming that the response of the chemoreceptors to anoxia is the same in the hypoglycemic and in the normal state.

<sup>5</sup> Kraines, S. H., and Gellhorn, E., *Am. J. Psychiatry*, 1939, **97**, 1067.

<sup>6</sup> Gellhorn, E., *Arch. Neurol. Psychiat.* Chicago, 1938, **40**, 127.

<sup>7</sup> Heymans, C., and Bouckaert, J. J., *Fræbn. Physiol.* 1939, **41**, 28.

<sup>8</sup> Modell, W., *Science* 1939, **89**, 349.



the rise in blood pressure as a response to anoxia, or changed the fall of the pressure during anoxia into a rise. As narcosis became deeper the blood pressure level often fell, so that the blood pressure rise as a response to anoxia, though larger, often failed to reach as great a height as a similar rise during light narcosis. In Fig 1 are shown 3 responses to anoxia in an experiment which does not have this disadvantage. A cat of about 3 kg body weight was given artificial respiration and at 10-minute intervals the animal was caused to breathe nitrogen instead of air for a period of 1 minute. The response in very light narcosis (100 mg chloralose) was a very marked fall of the blood pressure accompanied by a decrease of the heart rate (A). Three previous periods of anoxia in this state of narcosis had given almost identical curves. After injecting another 100 mg of chloralose the blood pressure response (B) consisted of a rise followed by a fall. In curve A also, a suggestion of this initial rise can be seen. After the injection of another 100 mg of chloralose the initial rise was still larger and the following fall in blood pressure had diminished materially (C). The blood pressure was practically the same at these 3 levels of narcosis. These experiments suggest that a moderate depression of the functions of the c n s favors a blood pressure rise as a response to anoxia. It is likely that this is caused by a suppression by the narcosis of reactions counteracting a marked rise in blood pressure either caused directly by the anoxia or indirectly by a slight rise in blood pressure (*e g* the carotid sinus pressure reflex). It has to be assumed that these latter reactions are depressed more markedly by the narcosis than are the reactions causing the rise in blood pressure.

It is well known that hypoglycemia causes a depression of the functions of the c n s, which can in severe conditions lead to coma. Less severe hypoglycemia also depresses the functions of the c n s as has been shown by Wiedeking<sup>9</sup> for cortical functions. It is therefore quite possible that the combined effects of hypoglycemia and narcosis caused in the experiments of Gellhorn, Ingraham and Moldavsky, a depression of the c n s to such a degree that anoxia brought about a marked rise in blood pressure because the reactions counteracting this rise were depressed. To examine this conception the effect of hypoglycemia on one of the reactions counteracting a marked rise in blood pressure was investigated, namely the depressor reflex in the rabbit. Two to 3 hours after the intravenous injection of 10 to 15 units of insulin per kg body weight in the fasting animal, the depressor nerve was isolated and the carotid artery arranged for

<sup>9</sup> Wiedeking, J, *Z ges Neurol*, 1937, **150**, 417

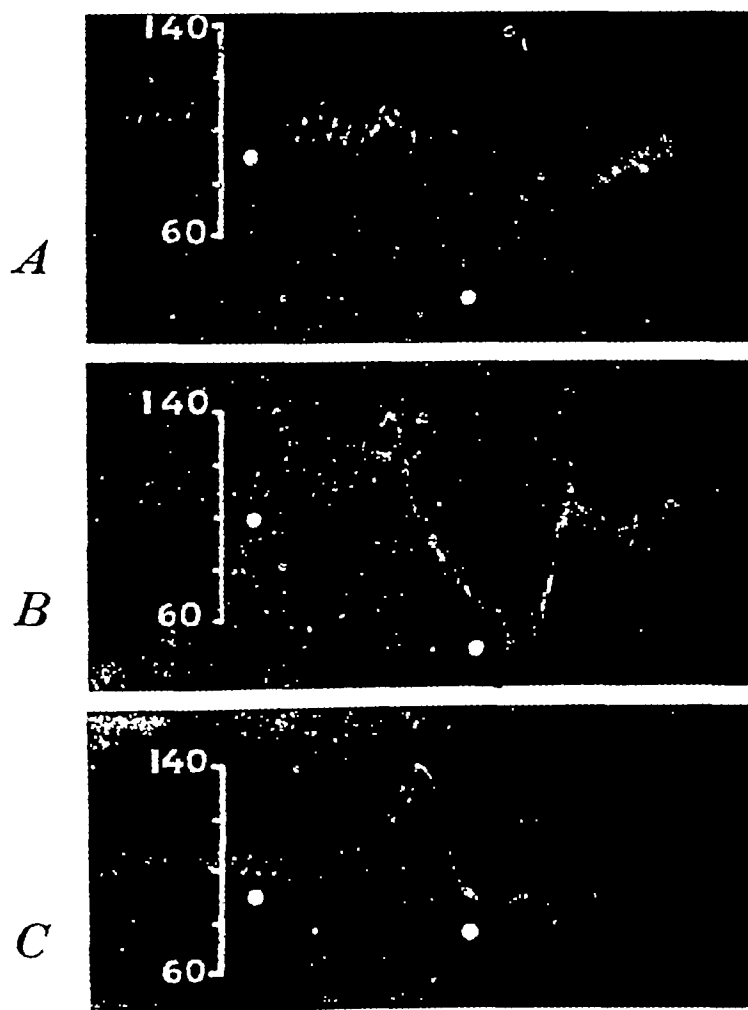


FIG 1

Three blood pressure responses to anoxia (nitrogen for 1 minute) at 3 levels of narcosis in a cat of 3 kg bodyweight. The white dots indicate the beginning and end of introducing nitrogen into the apparatus for artificial respiration. "A" after administering 100 mg of chloralose; "B" after a total of 200 mg and "C" of 300 mg of chloralose. Time intervals: 1 second.

blood pressure recording. The animals usually were in a hypoglycemic coma and showed occasional convulsions. Chloralose or nembutal was used as narcotic. The blood sugar was determined before and after the intravenous injection of 3 to 5 gr glucose. The depressor nerve was stimulated faradically with maximal stimuli



Before injecting glucose the depressor nerve was stimulated repeatedly, no large fluctuations of the responses were observed. In all the cases presented in Table I the fall in blood pressure during 10 sec of depressor stimulation was larger after administering glucose than during hypoglycemia. It takes about 20 minutes for this effect of the glucose injection to fully develop. In some cases (2 and 5) the stimulus did not cause any reaction during hypoglycemia, after injection of glucose, however, the same stimulus caused an appreciable fall. It can be concluded that this reflex, like many other reactions of the c n s, can be depressed by hypoglycemia.

On repeating Gellhorn, Ingraham and Moldavsky's experiment on the rabbit we found in part of the cases no marked blood pressure rise caused by anoxia, though the blood sugar level was found to be quite low. It is possible that this is characteristic for the rabbit since Gellhorn, Ingraham and Moldavsky, working on the dog, did not report this inconstancy. It must be assumed that in these cases the combined effect of hypoglycemia and narcosis did not depress markedly the reactions counteracting a blood pressure rise. In agreement with this the depressing effect of hypoglycemia on the depressor reflex was found only in part of the experiments, in the others the injection of glucose in the hypoglycemic animal caused no change in this reflex.

This explanation of the marked rise in blood pressure as a response to anoxia during hypoglycemia, as a depression of the functions of the c n s is quite in keeping with the usual depressing effect of hypoglycemia on the c n s.

*Conclusion* The large blood pressure rise due to anoxia, observed during hypoglycemia may be caused by a severe depression by the combined effect of narcosis and hypoglycemia of those reactions which counteract a marked rise in blood pressure, the reactions causing this rise in blood pressure being less severely depressed.

## Bleeding Induced in Uterine Mucosa of the Rabbit by Estrogenic Hormone

BERNHARD ZONDEK

*From the Laboratory of the Gynecological Obstetrical Department of the Rothschild Hadassah University Hospital Jerusalem*

By using gonadotropic hormone it is possible to produce in the rabbit a profuse bleeding which roots up the entire uterine mucosa<sup>1</sup>. The blood penetrates through the lifted areas of the epithelium into the uterine cavity and finally escapes into the vagina. This bleeding is observed especially after intravenous administration of the hormone and only rarely after subcutaneous administration. The most suitable dosage proved to be 100 RU gonadotropic hormone daily for 5 days. The bleeding appears on the 6th day. This effect was obtained with gonadotropic hormone from pregnancy urine or pregnant mare serum. The immature rabbit, weighing 1000-1400 g, is more suited for these experiments than when sexually mature. If the extirpated uterus is inspected against a strong light the blood can be recognized in the uterus with the naked eye but the macroscopical findings were not solely relied upon. Each preparation was examined histologically. In some cases the blood was found distributed over wide areas, sometimes there were only circumscribed spots. To be sure that bleeding had not been artificially produced by handling, the abdomen was opened, then filled with 4% formalin and the uterus was removed several hours later. It was possible to induce proliferation, and in some cases, progesterational transformation in the mucosa uteri by administration of gonadotropic hormone for 5 days. In no instance was the bleeding found in the progesterationally altered mucosa but only in the proliferative one. In the same uterine horn different areas may show different stages of development. Even in such cases the bleeding was always found in the proliferatively developed areas. From this we may deduce that progesterone counteracts the bleeding effect.

Bleeding could not be obtained in the castrated immature animal by the use of gonadotropic hormone, so it was obvious that the effect was produced *via* the ovary. The question now arose which of the two ovarian hormones was responsible for the production of the bleeding. The corpus luteum hormone could be eliminated, since no bleeding was ever encountered in the progesterational mucous mem-

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<sup>1</sup> Zondek, B, *J of Obst and Gynaecol of the Brit Emp*, 1938, 45, 1

brane, and experiments conducted with progesterone had also negative results. It is, therefore, the estrogenic hormone which should be considered in this connection. We injected into immature rabbits 100 IU of estrone in aqueous solution daily for 5 days. There was in some cases slight circumscribed bleeding, but we did not find the marked bleeding which spreads under the entire mucosa, eventually penetrating through the epithelium into the cavum uteri, as with gonadotropic hormone. Since it was not possible to produce bleeding of the uterine mucosa with either of the two ovarian hormones (estrone, progesterone) we suggested a third ovarian factor, hitherto unknown, which might be stimulated by gonadotropic hormone.<sup>1</sup> This assumption was found unnecessary, for it has been found possible to achieve the bleeding effect with estrogenic hormone, if a special experimental procedure is adhered to, taking into account the following factors:

- (1) Dosage
- (2) Interval between administration of hormone and examination for the bleeding effect
- (3) Mode of application

The most suitable dosage was 2 injections of 500 to 750 IU of estrone given at intervals of 12-24 hours. It is of prime importance that a powerful hormonal stimulus should be given in the course of one day, then wait several days. Usually the bleeding appears after an interval of 5 days. The most effective mode of application is by the intravenous route. The intravenous injection of estrogenic hormone is practicable in aqueous solution prepared in the following way: Dissolve 10 mg of crystallized estrone in a small quantity of absolute alcohol, add 1 cc of n/NaOH solution, after some time add water to 100 cc so that the estrone is dissolved in n/100 NaOH. The alcohol is then evaporated *in vacuo*. One cc of this solution contains 100 IU of estrone.

The experiments were performed with 13 immature rabbits. When estrone was injected subcutaneously in oily solution hyperaemia but no bleeding was observed (R 604 and 606). When the hormone was spread over 4 days (R 591) or when too large doses were used (3000 IU) bleeding was not obtained (R 576, 579, 597). Adhering to the above described experimental procedure bleeding appeared in 5 of the experimental animals (Table I).

The estrone-produced bleeding is identical with that produced by means of gonadotropic hormone yet does not seem to occur so regularly. The vessels show lacuna-like dilation the blood penetrates the mucosa lifts the epithelium and enters the uterine cavity and

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<sup>1</sup> Zondek, B, *J of Obst and Gynaecol of the Brit Emp*, 1938, 45, 1

tion It has been shown<sup>2</sup> that the uptake of water in the Brunn reaction is 2 to 3 times as great in the summer as in the winter months in this locality No exogenous factor was found to account for this seasonal variation which was tentatively ascribed to variation in some endogenous mechanism<sup>2</sup>

Zahl<sup>3</sup> has reported that there is a seasonal variation in the histological picture of the pars distalis of the frog with more acidophilic cells in the winter than in the summer The acidophilic cells of the buccal lobe are believed to elaborate growth hormone and possibly other hormones such as the thyrotropic and gonadotropic factors<sup>4, 5</sup> We therefore considered it possible that an excessive production of anterior pituitary principles might have accounted for the depression of the Brunn reaction in our winter frogs That anterior and posterior pituitary hormones antagonize each other upon certain phases of mammalian water balance has been known since the initial experiments of Harvey Cushing and his colleagues, von Hann and others<sup>6</sup> Elmer writing from Lwów quotes Carter as stating that a "Ringer extract of the whole pituitary produced the winter condition in a summer heart" of frogs<sup>7</sup> Biasotti<sup>8</sup> found that an extract of the anterior part of the pituitary body had no immediate effect on the body water of a South American amphibian, *Leptodactylus ocellatus*

If anterior pituitary hormones account for the winter depression of the Brunn reaction, they may act in one or both of two ways, either by directly neutralizing the water balance component of pituitary (posterior lobe) extract or by so altering the receptor cells upon which this extract acts that it cannot effect as great an uptake of water into the body We wish to report experiments bearing upon the first possibility Using the same technic as in previous studies of the Brunn reaction<sup>2</sup> we injected into frogs in water pituitary (posterior lobe) extract without and with several commercial anterior pituitary, human pregnancy urine and pregnant mare serum preparations, the latter in doses corresponding by weight to 1 to 1000 times

<sup>2</sup> Boyd, E M, Mack, E G, and Smith, A E, *Am J Physiol* 1939, **127**, 328

<sup>3</sup> Zahl P A, *Proc Soc Exp Biol and Med*, 1935, **33**, 56

<sup>4</sup> Van Dyke, H B, *The Physiology and Pharmacology of the Pituitary Body* Univ of Chicago Press, 1936, and Vol 2, 1939

<sup>5</sup> Symposium *The Pituitary Gland* Williams and Wilkins Baltimore, 1938

<sup>6</sup> Fisher C, Ingram W R, and Ranson S W, *Diabetes Insipidus and the Neurohormonal Control of Water Balance*, Edwards Bros, Ann Arbor 1938

<sup>7</sup> Elmer A W, *Iodine Metabolism and Thyroid Function* Oxford Univ Press London, 1938

<sup>8</sup> Biasotti, A, *Compt rend Soc de biol* 1923 **88** 361



TABLE I.

Bleeding of the Uterine Mucosa of the Rabbit Induced by Estrogenic Hormone

Animal (rabbit) No	Preparation	Dosage (IU)	Interval between injections	Mode of adminis- tration	Bleeding	Day when uteri were examined
565	Estrone aqu	2x750	1 day	i v	+	4
566	"	2x750	1 "	i v	+	5
569	"	2x750	1 "	i v	+	4
588	"	2x750	1 "	i v	+	5
589	"	2x750	1 "	i v	+	5
605	Estrone ol	2x500	1 "	s c	+	5
567	Estrone aqu	1x750	—	i v	—	3
576	"	3x1000	18 hr	i v	—	5
579	"	3x1000	18 "	i v	—	5
591	"	4x300	4 days	i v	—	5
597	"	3x1000	18 hr	i v	—	5
604	Estrone ol	2x250	1 day	s c	Hyperemia	5
606	"	2x750	1 "	s c	"	5

vagina The anatomical picture greatly resembles that found in bleedings from a proliferatively developed mucosa in humans

The sexual cycle, in the rabbit, is without bleeding That it is possible to produce bleeding as in humans and monkeys, may provide the opportunity of studying the mechanism of the uterine bleeding in rabbits

*Summary* With estrone bleeding may be induced in the uterine mucosa of the rabbit The blood penetrates through the lifted epithelial areas and eventually enters the uterine cavity and vagina This effect may be obtained by two injections of 500 to 750 IU of estrone given intravenously during 12 or 24 hours Bleeding appears after 4-5 days

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## Anterior Pituitary Extracts and the Brunn Reaction in Frogs

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When pharmacopoeial extracts of the "posterior pituitary gland" are injected into frogs immersed in water there follows an increase in body weight due to an uptake of water which lasts 3 to 6 hours at room temperature This reaction was first investigated by Fritz Brunn at Prague<sup>1</sup> and we shall refer to it simply as the Brunn reac-

<sup>1</sup> Brunn, F, *Zeit f exp Med* 1921, 25 170

## Studies on the Bovine Electrocardiogram I Electrocardiographic Changes in Calves on Low Potassium Rations \*

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Four calves were placed on a semipurified ration which analyzed 0.10-0.12% potassium. Four control calves were given the same ration except that potassium was added to bring the level of this constituent to 0.35%. The animals were placed on this ration at 160 days of age. Previous to this they received whole milk and a small amount of hay and grain.

Electrocardiograms were usually taken at monthly intervals but for some periods more frequently. The electrodes which were of the German silver plate type were placed on the upper part of the 2 fore limbs and just above the hock on the left hind leg. The areas on which the electrodes were placed were clipped, cleaned and covered with electrode paste applied with vigorous rubbing.

Serum potassium determinations were made on all calves at 2-week intervals more often when deemed necessary by a modification of the titrimetric method of Shohl and Bennett <sup>1</sup>.

Plasma calcium, phosphorus and magnesium determinations were carried out at weekly intervals in order to make certain that the ration was not deficient in these elements.

*Blood Changes. Calf C 394.* On the 270th day of the experimental period this calf had a serum potassium value of 17.0 mg %. Seventy days later this value had fallen to 10.2 mg % and for a period of about 60 days it remained below 13.5 mg %. On the 350th day of the experiment the potassium in the ration was increased to 0.20% for a period of 130 days after which the potassium was again reduced. During this 130-day period the serum potassium level increased to 18.0 mg % and it has since varied between 15 and 18 mg % in spite of the reduction of potassium in the ration.

*Calf C 395.* On the 270th day of the experimental period the serum potassium level of this calf was 21.9 mg %. On the 340th day it had decreased to 11.3 mg % and it remained below 14.0 mg % for a period of about 60 days. The potassium of the ration was then

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<sup>1</sup> Shohl, A. T., and Bennett, H. B. *J. Biol. Chem.* 1928 78: 643.

the average recommended single injected human dose. These preparations used included Gonan and Serogan (B D H), Antophysin (Winthrop), Antuitrin-G (P D & Co) and the gonadotropic, thyrotropic and lactogenic fractions marketed by Ayerst, all of these preparations being generously provided us by the companies concerned. A grant which defrayed part of the expenses of the investigation was provided by Parke, Davis and Co through Dr E A Sharp.

None of these preparations had any effect upon the Brunn reaction in the doses employed. To illustrate this, the percentage uptake of water was averaged in all groups of frogs receiving the same human dose equivalent of the preparations and 1 international unit of pituitary (posterior lobe) extract per 10 g body weight giving a total of 96 frogs to each group and the mean values so obtained have been compiled in Table I. Similar experiments made with lower doses of pituitary (posterior lobe) extract down to 0.1 units per 10 g yielded similar negative results. These results indicate that that fraction of pituitary (posterior lobe) extract which produces the Brunn reaction is not directly neutralized by any of the anterior pituitary or anterior pituitary-like hormones present in the preparations used.

It seemed unlikely that any of these anterior pituitary or anterior pituitary-like preparations could of themselves affect body water during the 5-hour interval after their injection when body weight was being measured. To be sure of this, we injected them without pituitary (posterior lobe) extract and in a dosage range similar to that used above and found in some 500 frogs that changes in body weight were not significantly different from those in controls.

*Conclusion.* A number of anterior pituitary and anterior pituitary-like preparations were found to have no effect during 5 hours after their injection into frogs in water either on normal body water or upon the Brunn reaction.

TABLE I

Uptake of Water by Leopard Frogs in Water Injected with Pituitary (Posterior Lobe) Extract with and without Increasing Doses of Anterior Pituitary Preparations (AP)

Group	% uptake of water (Mean 96 frogs)			
	2 hr	3 hr	4 hr	5 hr
Pituitrin alone	14.1	15.6	13.5	9.9
" + equivalent human dose AP	13.1	15.4	13.1	9.9
" + 10X equivalent human dose AP	11.8	15.2	13.8	10.4
" + 100X " " " "	10.0	14.4	12.8	9.4
" + 1000X " " " "	10.3	15.1	13.5	10.8

The calcium, phosphorus and magnesium values of the plasma remained within normal limits in both experimental and control animals

*Electrocardiographic Changes* Three of the 4 experimental animals showed pronounced changes in the electrocardiogram. The other (C 410) failed to show any change possibly because the serum potassium did not fall so low or remain at as low a level for so long a period in this calf as in the others.

The outstanding change in the electrocardiograms of these calves consisted in a pronounced increase in the duration of the QRS. The Q-T interval, also, increased, but this increase was obviously due mainly to the broadened QRS deflection. The P-R interval was within normal limits at all times. In 2 calves the QRS interval increased to more than 0.16 second and in one calf it exceeded 0.20 second. These QRS intervals are approximately twice as long as those displayed by the electrocardiograms of normal mature cattle and the electrocardiograms of the control animals. In a group of 97 animals, 60 of which were over 2 years of age, the average duration of QRS was 0.094 second and its maximum duration was 0.12 second (Alfredson unpublished data). Barnes, Davis and McKay,<sup>2</sup> who studied

TABLE I  
Heart Rate and Duration of QRS in Experimental Calves

C 394			C 395			C 409			C 410		
Age	Rate	QRS	Age	Rate	QRS	Age	Rate	QRS	Age	Rate	QRS
175	43	10	177	72	08	26	107	06	32	120	06
216	42	12	210	44	10	77	75	06	83	100	06
253	58	08	255	42	08	105	84	06	111	94	06
260	65	09	304	125	06	176	112	07	172	94	06
309	79	12	305	107	08	189	88	07	185	107	08
330	100	12	381	100	10	211	88	08	207	79	08
386	79	10	403	84	12	224	100	06	220	88	08
408	84	10	416	63	11	237	88	08	233	63	08
421	125	10	429	50	12	299	63	14	272	60	07
434	52	10	461	48	13	300	88	14	295	43	08
460	47	10	482	52	12	301	92	12	309	43	08
484	65	10	505	50	16	302	88	08	359	68	08
485	65	08	555	47	16	306	77	08	437	72	08
486	65	10	633	84	18	308	79	10	479	50	08
490	56	11				313	42	09	528	75	09
492	56	12				363	72	12	563	72	09
497	32	12				441	57	12	595	75	08
545	52	12				483	64	19	623	72	10
623	75	17				532	79	14			
665	63	18				557	68	16			
714	63	20				591	70	15			
749	56	24				619	60	18			
773	48	20									
801	50	24									

<sup>2</sup> Barnes, L. K., Davis, G. K. and McKay, C. M. *Cornell Vet.* 1935 38 16

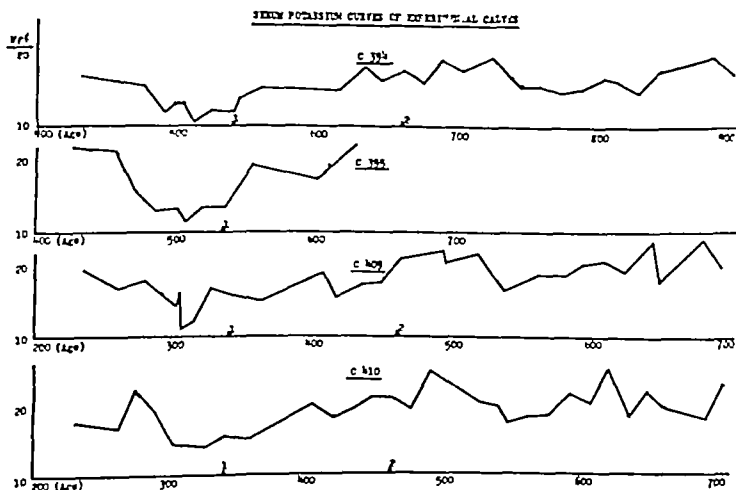
increased to 0.20%. The serum potassium promptly increased to 19.5 mg % and had reached a level of 22.4 mg % when the animal was slaughtered.

**Calf C 409** From a level of 19.5 mg % on the 75th day of the experimental period the serum potassium decreased to 10.7 mg % on the 140th day. A low level was maintained for about 90 days. On the 175th day the potassium of the ration was increased to 0.20% and it was kept at this level for 130 days. During this time the serum potassium values returned to normal and have remained so in spite of the reduction of the potassium in the ration to 0.10% at the end of the 130-day period.

**Calf C 410** The changes in this calf closely paralleled those of C 409. The serum potassium value of 17.6 mg % on the 70th day decreased to 13.8 mg % on the 160th day. Low values were maintained for about 60 days. During the 130-day period in which the potassium of the ration was increased to 0.20%, the serum potassium rose to 21.0 mg % and has since remained at or near this level.

The serum potassium values of these 4 calves are shown graphically in Fig. 1.

The serum potassium values for the 4 control calves averaged respectively  $21.4 \pm 1.2$  mg %,  $20.4 \pm 1.5$  mg %,  $20.5 \pm 1.4$  mg % and  $21.6 \pm 1.8$  mg %.



- 1 Potassium of ration increased  
2 Potassium of ration reduced to previous level

FIG. 1  
Serum potassium curves of the experimental calves

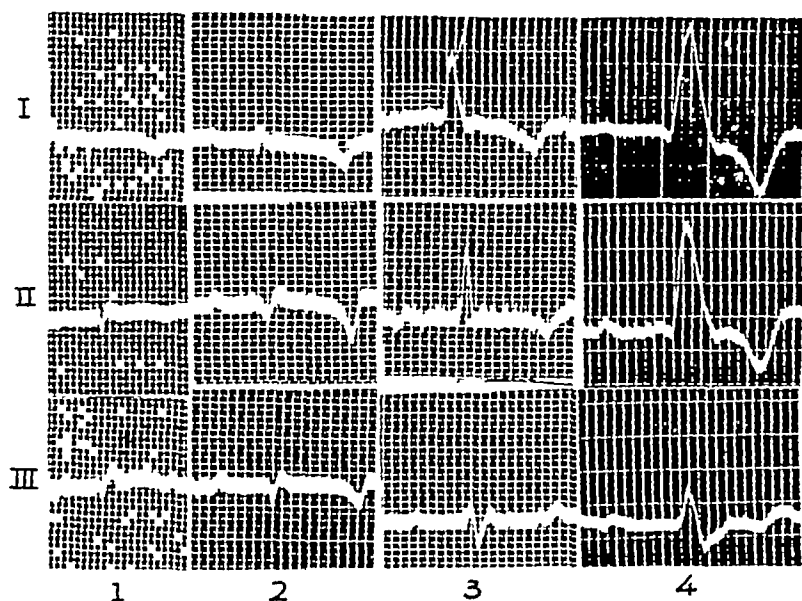


FIG 2

Electrocardiogram changes typical of the experimental calves 1 Control tracing 2 Tracing at 460 days of age 3 Electrocardiogram at 492 days 4 Electrocardiogram taken at 750 days of age

ascribed to bundle branch block. A preliminary examination of various sections of the Purkinje system of right and left ventricles revealed pathological changes which are not restricted to any one particular region of the heart. A later report will describe these changes in further detail.

Arrhythmias appeared at irregular intervals both during the experimental period and during the preexperimental period. They were perhaps more frequent during the former than during the latter but the difference was not great enough to be of certain significance.

This work is part of a project in which the Department of Dairy Husbandry and Experiment Station Chemistry is cooperating. The assistance of the staff members of these two departments, Dr C F Huffman, Mr C W Duncan, Dr L H Greathouse, and Miss L I Butler, is gratefully acknowledged. The criticisms and suggestions of Dr F N Wilson, University Hospital, Ann Arbor, Michigan, during the preparation of the manuscript are also gratefully acknowledged.

TABLE II  
Heart Rate and Duration of QRS in Control Calves

C 387			C 392			C 419			C 420		
Age	Rate	QRS	Age	Rate	QRS	Age	Rate	QRS	Age	Rate	QRS
217	50	06	214	72	08	56	150	06	67	54	06
247	94	06	219	54	07	69	75	06	90	58	07
298	68	06	250	88	07	114	55	07	120	94	07
340	68	07	286	91	08	135	68	07	154	65	07
361	57	08	293	97	08	174	75	07	232	68	07
417	58	09	300	100	08	208	120	08	274	100	08
439	68	07	342	75	08	286	88	08	323	88	08
454	75	08	363	68	08	318	100	07	358	88	08
506	60	07	398	75	09	367	115	08	392	84	07
529	63	10	419	68	08	402	65	07	420	79	08
559	54	08	441	65	09	436	84	08	517	72	08
671	56	11	467	65	08	464	88	08	558	79	08
713	65	10	506	68	09	561	65	09			
763	57	12	542	60	09	602	72	09			
			569	60	08						
			604	42	09						
			682	72	08						
			724	94	09						
			773	63	10						

normal calves ranging from 12-589 days of age, reported QRS intervals similar in length to those observed by Alfredson

The changes in duration of the QRS complex and the heart rate are shown in Table I. Table II gives similar data for the 4 control calves

The increase in duration of QRS began in 2 of the calves before the lowest levels of serum potassium were reached. In the third calf the increase began to appear when the lowest blood values were observed. The intervals became progressively greater and did not subsequently decrease even though the serum potassium values returned to approximately normal levels when the potassium content of the ration was raised.

The increase in the duration of the QRS complex was accompanied by equally striking changes in its contour and voltage. These changes are illustrated in Fig. 2, in which the electrocardiograms of calf C 394 are reproduced. The changes which occurred in the other animals were similar in a general way but varied in detail. It will be noted that the changes were progressive, and that the final record<sup>4</sup> resembles the human and canine curves which depict left bundle-branch block. It has been shown, however,<sup>3</sup> that section of the left branch of the His bundle in the calf produces relatively minor changes in the QRS interval and curves here in question cannot therefore be

<sup>3</sup> Alfredson, B. V., and Sykes, I. F., *Proc. Soc. Exp. Biol. and Med.* 1940, 43, 580

ments of this kind in mature cattle, we have used young calves. For comparison, a number of experiments have been performed on dogs.

*Procedure* In all instances sodium pentobarbital<sup>4</sup> was administered intravenously until surgical anesthesia supervened. The animals were held in the dorsal recumbent position. The thorax was opened and held as widely open as possible throughout the experiment by means of hook spreaders. In some of the earlier experiments the slit pericardium was stitched to the edges of the thorax, thus forming a cradle for the heart.<sup>†</sup> This procedure interfered with the manipulations necessary for efficient sectioning of branches of the His-bundle and was abandoned in favor of simply replacing the heart in the slit pericardial sac within the opened thorax. Section of the right or left bundle branch was carried out by the method described by Lewis.<sup>5</sup> Electrocardiograms were obtained before and after section. In 6 instances the presence of complete bundle branch block was proved by the onset of complete A-V block following a cut on the opposite side of the septum later shown to have transected the remaining bundle branch. In all cases the heart was removed at the end of the experiment, opened according to the method of Cardwell and Abramson<sup>1</sup> and examined to determine the location of the cuts. In view of the very clear demarcation of the right and left bundle branches in the ox heart, it is easy to determine in this way whether the bundle branches have been completely divided. Bundle branch block was successfully produced in 14 calves and in 10 dogs. Measurements of the QRS interval were made in lead II throughout as this is generally the most satisfactory lead in cattle.<sup>3</sup>

*Results and Discussion* The changes in the duration of QRS after section of the right or left bundle branch are recorded in Table I.

The average increase in duration of QRS on section of the right bundle branch (8 calves) was 0.013 sec, and the average increase after section of the left bundle branch (6 calves) was 0.005 sec. Right bundle branch block in dogs increased the duration of QRS 0.021 sec. Left bundle branch block increased the duration of QRS 0.029 sec. These are average values. The change in the duration of QRS in calves as compared to the change produced in dogs was therefore relatively insignificant.

Fig. 1 shows changes in the form of QRS typical of those produced by section of right and left bundle branches in calves and in

<sup>4</sup> Hafkesbring, R., and MacCalmont, W. *J. Pharm. and Exp. Ther.* 1938 **64**, 43.

<sup>†</sup> In the first 8 bovine subjects only 2 of which (B4 and B7) were included in the data.

Lewis, T. *Phil. Trans. Roy. Soc.*, 1916, **207B**, 254-267.



## Studies on Bovine Electrocardiogram II Bundle Branch Block \*

BERNARD V ALFREDSON AND JOSEPH F SYKES (Introduced by  
F N Wilson )

*From the Department of Physiology and Pharmacology, Michigan State College,  
East Lansing*

Recent work by Cardwell and Abramson<sup>1</sup> and Abramson and Margolin<sup>2</sup> has shown that, in some species at least, the arborizations of the Purkinje network of the heart are not confined to the subendocardium as is generally assumed. By means of an injection technic they demonstrated that in the ox heart branches of the Purkinje network pierced the ventricular septum and penetrated the outer ventricular walls nearly, if not quite, to the epicardium. The branches which pierced the septum made connections with the Purkinje network of the other side. Histological studies disclosed Purkinje tissue in all muscle layers of the ventricular septum and outer ventricular walls of the hearts of dogs, sheep and pigs, but in these animals the differentiation between the Purkinje tissue and the ordinary muscle was less pronounced than in the ox heart. For this reason and because the injection method failed in these animals the distribution of the Purkinje tissue could not be worked out as completely in them as in the ox. Electrocardiographic observations by one of us<sup>3</sup> suggest that contrary to the conclusions of the authors quoted,<sup>1, 2</sup> there may be major differences between the intraventricular conducting system of the ox heart and that of the canine heart.

In comparison with the canine and with the human heart, the ox heart is very much heavier and its walls very much thicker. It would be expected, therefore, that the QRS interval of the ox would be much longer than that of the dog or of man. In a study of the normal electrocardiogram of dairy cattle it was found that this was by no means the case. In a group of 97 cattle the mean duration of QRS was 0.09 sec with a minimum of 0.06 sec and a maximum of 0.12 sec.<sup>3</sup> These findings suggested that it would be worthwhile to determine the effect of section of the bundle branches of the ox heart upon the bovine electrocardiogram. Since it is difficult to carry out experi-

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\* Journal article No. 423 (n.s.) from the Michigan Agricultural Experiment Station

<sup>1</sup> Cardwell, J. C. and Abramson, D. I., *Am. J. Anat.* 1931, **40**, 769

<sup>2</sup> Abramson, D. I., and Margolin, S., *Am. J. Anat.*, 1936, **70**, 250

<sup>3</sup> Alfredson, B. V., unpublished data

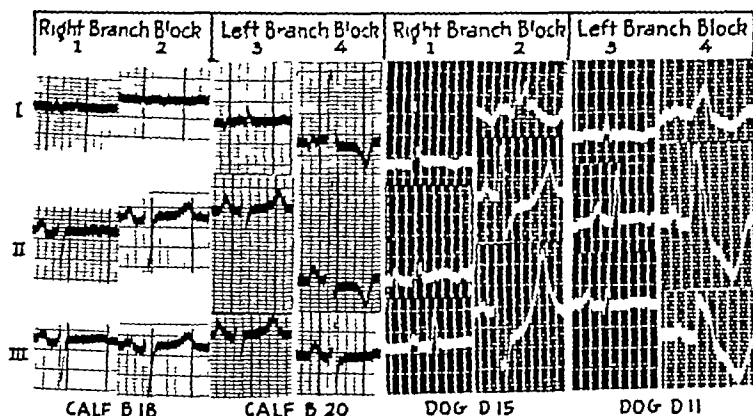


FIG 1

Electrocardiographic changes typical of those produced by section of the right and left bundle branches in calves and dogs. No 1 and 3 are control records. No 2 and 4 are records taken after section of the right or left bundle branches.

dogs. The canine left and right branch block curves are similar in form to those described by Lewis<sup>5</sup> and are very different from the control curves. The right branch block curves of the calves, however, were scarcely distinguishable from the control curves in the majority of instances. In some instances there was a slight increase in the size of S and in the voltage of T in leads II and III.

In comparison with the controls the left branch block curves of the calves showed much greater changes in the form of the ventricular complex but the character and magnitude of these changes varied greatly in different experiments. The form of the control curves was quite constant ‡. The deflections of Lead I were extremely small. In the other leads QRS consisted of an upright deflection (R) followed by a downward deflection (S). The relative size of R and S was variable but in most instances the latter was larger than the former. The T-waves were usually upright but of small voltage. After section of the left bundle branch the voltage of the largest QRS deflection was greater than in the control. In one instance QRS was represented by a large downward deflection in Lead I and a large upward deflection in Leads II and III. In this instance T<sub>2</sub> and T<sub>3</sub> were large and inverted. In 2 instances large R-waves and large inverted T-waves appeared in Leads I and II. In another experiment the changes were similar to those just described but less pronounced, in another the R deflection became larger and the T-wave became inverted in Leads II and III but the deflection in Lead I remained

‡ This uniformity is not seen in electrocardiograms of the normal bovine subject when taken in the standing position.

TABLE I  
Duration of QRS in Lead II Before and After Section of Right or Left Branches of the His Bundle

Right Bundle Branch Block					Left Bundle Branch Block				
Animal No	Age (days)	Duration of QRS (sec)			Animal No	Age (days)	Duration of QRS (sec)		
		Before section	After section	Increase			Before section	After section	Increase
Calves									
B 7	14	05	06	01	B 4	7	04	05	01
B 9	21	05	06	01	B19	44	04	05	01
B10	21	05	06	01	B20	3	05	05	
B12	14	055	07	015	B21	28	05	05	
B13	14	055	07	015	B22†	28	055	065	01
B15	14	055	08	025†*	B23	3	05	05	
B17	240	06	07	01					
B18	46	05	06	01					
Mean		053	060	013			047	052	005
Dogs									
D 5	8	08	10	02	D 2		05	09	03
D 7	05	08	08	03	D11		055	08	025
D 8	04	07	07	03	D12		05	08	03
D13	055	065	065	01					
D14	065	08	08	015					
D15	06	08	08	03					
D16	055	07	07	015					
Mean		056	077	021			051	08	029

\* The exact QRS interval was difficult to determine in this instance because the end of the QRS group was ill defined  
 † Failure to record lead II in the control tracing due to insufficient opening of the camera shutter necessitated the taking of data from lead III in this subject

Fermentation of Pyruvic Acid by *Clostridium botulinum*\*

C E CLIFTON

From the Department of Bacteriology and Experimental Pathology, Stanford University, California

It has been reported<sup>1</sup> that the fermentation of glucose by Types A and B *Clostridium botulinum* differs from the majority of bacterial fermentations in that ethyl alcohol and carbon dioxide are the main products of the fermentation, only small amounts of acetic and lactic acids together with traces of hydrogen being formed. This study has been extended to include the fermentation of pyruvic acid, which may be an intermediate compound in the fermentation of glucose and also in the degradation of amino acids such as alanine, by *Cl botulinum*.<sup>2</sup>

The experiments reported below were carried out with washed suspensions of Type A (E-43) *Cl botulinum* although studies with Type B gave essentially the same results. Glucose-broth (800 ml) containing 0.1% Difco yeast extract was inoculated with 1.0 ml of a beef-brain culture of the organism and incubated for 20 hours at 37°C in a McIntosh and Fildes anaerobic jar. The culture was then centrifuged and the cells suspended in distilled water. The suspension was diluted with an equal volume of M/7.5 phosphate buffer and placed in the central chamber of Warburg vessels. A rapid stream of O<sub>2</sub>-free H<sub>2</sub> was passed through the vessels for 10 minutes and they were then equilibrated 10 minutes before tipping in the sodium pyruvate from the side-arm. CO<sub>2</sub> production was determined at 37°C by the Warburg technic, the initial and final bound CO<sub>2</sub> being determined following the addition of 10% sulfuric acid to the contents of separate Warburg vessels. In the semi-macro experiments, Warburg vessels of 40 ml capacity were employed with Clerici fluid in the manometers.

It was observed that pyruvic acid is rapidly decarboxylated by washed suspensions of *Cl botulinum*, Q<sub>CO<sub>2</sub></sub> values of 25-30 being observed under optimal conditions. Typical results over a pH range of 5.6 to 7.5 are reported in Fig. 1. Following correction of the results for CO<sub>2</sub> bound by the phosphate buffer it is apparent that the pH optimum lies near 6.0. Accordingly the majority of the ferment-

\* Aided in part by a grant from the Rockefeller Fluid Research Fund

<sup>1</sup> Clifton, C. E., *J. Bact.*, 1940, in press

<sup>2</sup> Clifton, C. E., *Proc. Soc. Exp. Biol. and Med.*, 1939, 40, 338

small In the remaining experiments the changes were very slight. Distinct notching of the larger QRS deflections did not occur in any experiment

Compared to the electrocardiographic changes produced by right and by left bundle branch block in man and in the dog, those produced by cutting either of the bundle branches of the calf's heart are extremely small and as regards the form of the electrocardiogram conspicuously variable This difference suggests that there is a decided difference in the distribution of the intraventricular conducting system between man and the dog, on the one hand, and the calf on the other The smallness of the QRS interval of the bovine electrocardiogram points in the same direction It is difficult to understand how the cardiac impulse can spread so quickly over the ventricular muscle of so large a heart if it spreads with approximately the same speed and in the same manner as in the human and the canine heart The pronounced increase in the QRS interval produced by bundle branch block in man and in the dog has been attributed to the slow spread of the cardiac impulse through the ordinary muscle of the ventricular septum The absence of a similar increase in the QRS following section of the bundle branches of the calf's heart suggests that in this animal the Purkinje networks of the 2 ventricles are connected by strands of specialized tissue which pierce the ventricular septum Penetration of the outer ventricular walls by Purkinje tissue would for similar reasons account for the smallness of the QRS interval of the bovine electrocardiogram in comparison with the size of the ox heart The differences between the branch block curves of the dog and those of the calf suggest that contrary to the conclusions of Abramson and his fellow workers the distribution of the Purkinje tissue is not similar in the two species, and that this tissue does not penetrate the ventricular septum of the canine heart The same conclusion may be drawn with respect to the human heart and the hearts of other species in which bundle branch block induces electrocardiographic changes of comparable magnitude

*Summary* Changes in the duration and form of QRS after section of the branches of the His-bundle are much less pronounced in calves than in dogs The difference between these two species is attributed to differences in the distribution of the intraventricular conducting system

The authors wish to express their thanks for the assistance and advice of Dr F N Wilson, University Hospital, Ann Arbor, Michigan, and Dr E T Hallman, Michigan State College, East Lansing The experimental animals were generously donated by the Dairy Department of Michigan State College

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<sup>1</sup> Clifton, C. E., *J. Bact.*, 1940, in press

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tations were carried out at this pH although similar results were obtained at pH 7.0. The fermentation of glucose differs from that of pyruvic acid in that the rate is much less dependent on the pH over a range of 5.6 to 7.8.

In preliminary experiments ethyl alcohol and acetic acid were identified as the main non-gaseous products of the fermentation. Alcohol was determined by oxidation with bichromate following distillation from alkaline solution and volatile acids by steam-distillation of the acidified residue. Ethyl alcohol was identified by the iodoform test and by the fact that acetic acid appears to be the only acid produced on oxidation with bichromate. This acid and that produced in the fermentation was identified as acetic by Duclaux distillations of the combined steam-distillates from several large-scale experiments. No gas other than  $\text{CO}_2$  was produced and the  $\text{CO}_2$  liberated by control suspensions was negligible. In general about 5% of the pyruvic acid was reduced to lactic acid during the course of the fermentation.

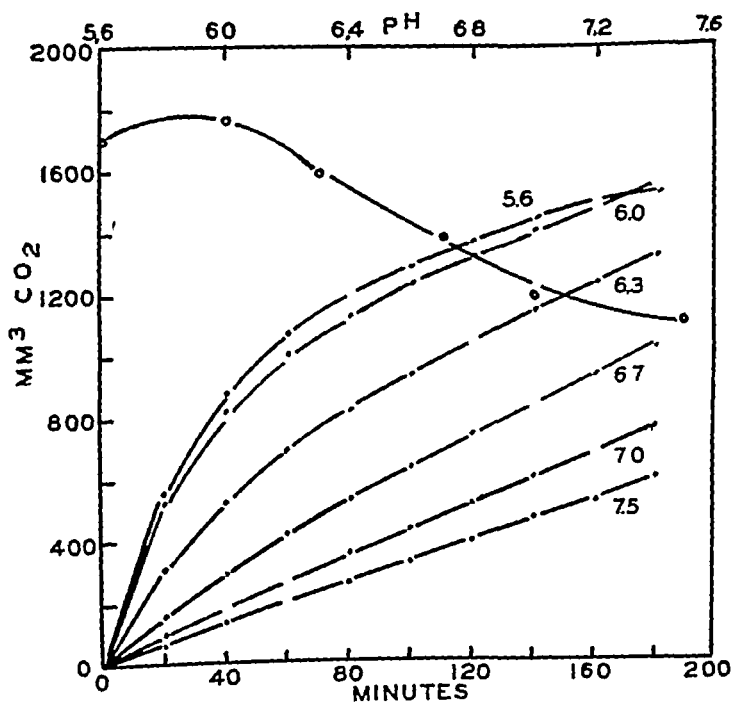


Fig. 1.

Influence of pH on the rate of  $\text{CO}_2$  liberation during the fermentation of pyruvic acid by *Cl botulinum*. O—O Total  $\text{CO}_2$  produced, corrected for bound  $\text{CO}_2$ , from 0.2 ml M/2 Na pyruvate in 180 minutes.

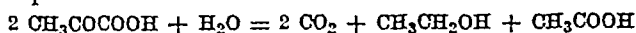
TABLE I.

Pyruvate fermented	mg 44	Mols/ mol pyruvate	mg 44	Mols/ mol pyruvate
Products				
CO <sub>2</sub>	19.4	88	19.8	.90
CH <sub>3</sub> CH <sub>2</sub> OH	12.3	53	13.0	.56
CH <sub>3</sub> COOH	13.2	44	13.8	.46
Total	44.9		46.6	

The results of typical duplicate semi-macro experiments at pH 6.0 are presented in Table I.

Similar results were obtained when the fermentation was carried out in an atmosphere of O<sub>2</sub>-free N<sub>2</sub>.

The results suggest that pyruvic acid is decarboxylated and the acetaldehyde produced dismutated with the production of equimolar quantities of ethyl alcohol and acetic acid. The above results are in fair agreement with this hypothesis since no correction was made for the small amounts produced by the organisms alone, the quantities being too small for accurate determination. In general the amounts of CO<sub>2</sub> recovered were somewhat less than the theoretical while the ethyl alcohol tended to run somewhat high. This tendency was also observed in macro experiments in which CO<sub>2</sub> production was determined by absorption of the liberated gas in standard Ba(OH)<sub>2</sub> solution. Possibly a small amount of CO<sub>2</sub> is utilized during the fermentation. It appears, therefore, that the fermentation of pyruvic acid may be represented as



If the fermentation of glucose proceeds by way of pyruvic acid, dismutation of acetaldehyde may account for the small amount of acetic acid produced, the bulk of the acetaldehyde being reduced to alcohol by a H-donor other than another molecule of acetaldehyde.



Utilization of Serine by *Clostridium botulinum* \*

C E CLIFTON

From the Department of Bacteriology and Experimental Pathology, Stanford University, California.

A number of amino acids are utilized by washed suspensions of *Clostridium botulinum*, for the most part in coupled reactions between pairs of different amino acids<sup>1, 2</sup>. For example, alanine is deaminatively oxidized to CO<sub>2</sub> and acetic acid by glycine or proline which are reduced to acetic acid or  $\delta$ amino-n-valeric acid, respectively. However, amino acids such as leucine and particularly serine appeared to be deaminated when present singly in washed suspensions of this organism. The utilization of serine has been studied in detail by the technic already described,<sup>3</sup> ammonia being determined by the method of Parnas as described by Niederl and Niederl<sup>4</sup>.

Serine is decarboxylated in the presence of washed suspensions of Types A or B *Cl. botulinum* at a rate ( $Q_{CO_2}$  of 7-10) approximately one-third of that observed with pyruvic acid ( $Q_{CO_2}$  of 25-30) as the substrate at optimal pH values for both reactions. Typical results of CO<sub>2</sub> production are presented in Fig. 1.

It is apparent that the pH optimum for the utilization of serine by *Cl. botulinum* lies on the alkaline side of neutrality, probably near pH 7.5. This is in marked contrast with the pH optimum of 6.0 observed with pyruvic acid as the substrate. In further studies it was observed that apparently only one optical form of serine is attacked at an appreciable rate, deamination and decarboxylation of *dl*-serine proceeding only approximately 50% to completion.

In semi-macro experiments in Warburg vessels of 40 ml capacity with Clerici fluid in the manometers the fermentation was studied in more detail. Ammonia, CO<sub>2</sub>, ethyl alcohol and acetic acid were identified as the chief products of degradation of serine. In these experiments the reaction was not allowed to go to completion due to the slow rate observed by the time approximately one-half of the serine had been utilized. Typical results on the utilization of serine

\* Aided in part by a grant from the Rockefeller Fluid Research Fund.

1 Clifton, C. E., *Proc. Soc. Exp. Biol. and Med.*, 1939, **40**, 338.

2 Clifton, C. E., *J. Bact.*, 1940, in press.

3 Clifton, C. E., *Proc. Soc. Exp. Biol. and Med.*, 1940, **43**, 585.

4 Niederl, J. B., and Niederl, V., *Micromethods of Quantitative Organic Elementary Analysis*, 1938, 51-59, John Wiley and Sons, New York.

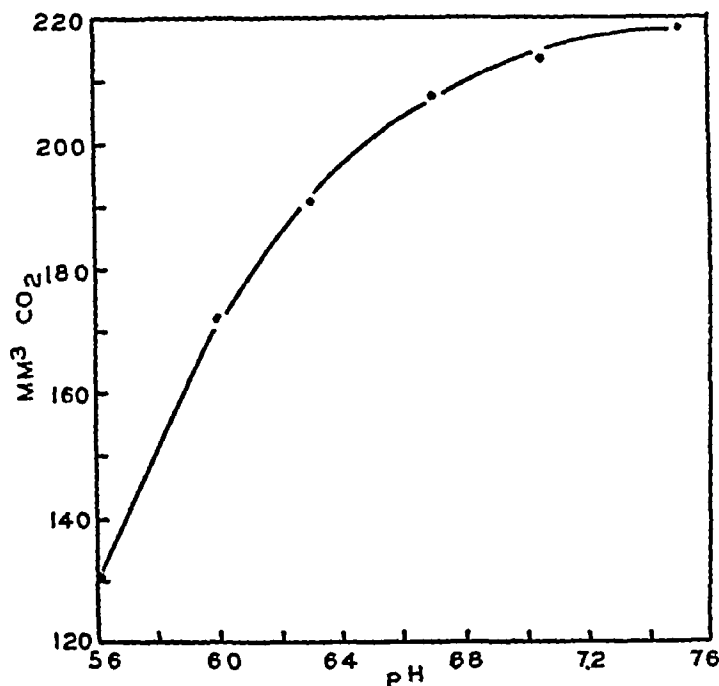


FIG. 1

Influence of pH on CO<sub>2</sub> production, corrected for bound CO<sub>2</sub>, by *Cl botulinum* from 0.2 ml M/10 dl serine in 90 minutes at 37°C

by washed suspensions of Type A *Cl botulinum* in M/15 phosphate buffer of pH 7.2 are recorded in Table I, the fermentation being carried out in an atmosphere of O<sub>2</sub>-free H<sub>2</sub>. Similar results were obtained in an atmosphere of O<sub>2</sub>-free N<sub>2</sub> and with washed suspensions of Type B *Cl botulinum*. The quantity of serine fermented is based on the NH<sub>3</sub> liberated during the course of the experiment.

The results suggest that serine is deaminated and rearranges to form pyruvic acid which is then fermented, or, that serine is de-

TABLE I.

	mg	Mols/mol serine	mg	Mols/mol serine
Initial serine	52.5		52.5	
Final serine	28.0		32.5	
Serine utilized	24.5		20.0	
Products				
NH <sub>3</sub>	3.98	1.00	3.25	1.00
CO <sub>2</sub>	8.98	0.87	7.17	0.86
CH <sub>3</sub> CH <sub>2</sub> OH	0.15	0.55	5.06	0.57
CH <sub>3</sub> COOH	7.20	0.51	6.00	0.53
Total	26.31		21.48	

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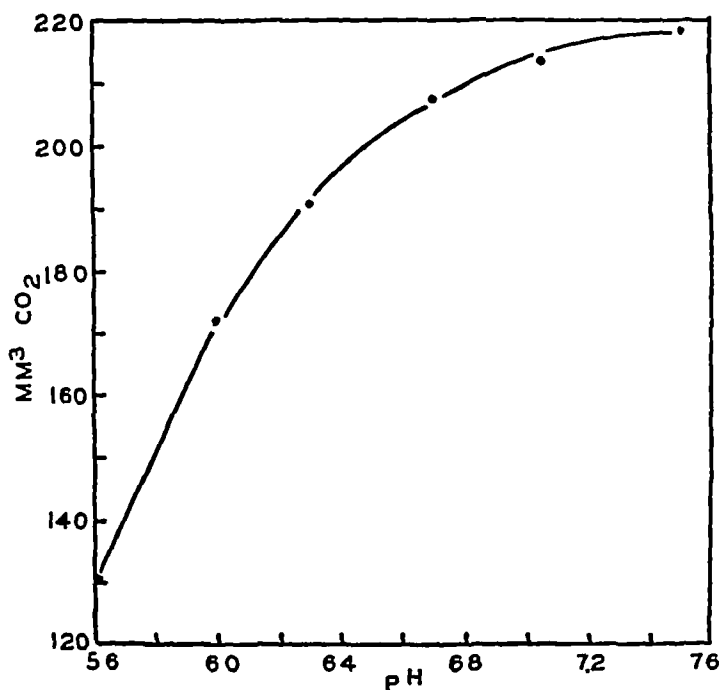


FIG 1

Influence of pH on  $\text{CO}_2$  production, corrected for bound  $\text{CO}_2$ , by *Cl botulinum* from 0.2 ml M/10 dl serine in 90 minutes at  $37^\circ\text{C}$

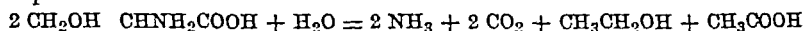
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$\text{CH}_3\text{CH}_2\text{OH}$	6.15	0.55	5.06	0.57
$\text{CH}_3\text{COOH}$	7.20	0.51	6.00	0.53
Total	26.31		21.48	

carboxylated and deaminated at the same time to yield acetaldehyde which is dismutated to form equimolar quantities of ethyl alcohol and acetic acid. If the reaction proceeds by way of pyruvic acid, deamination appears to be the controlling factor of the rate of utilization of serine as evidenced by the marked shift in pH optimum from 6.0 for pyruvic acid to the neighborhood of 7.5 for serine. These results, considering the experimental errors involved, suggest that the degradation of serine by washed suspensions of *Cl. botulinum* may be represented as



The closely related amino acid, alanine, is not attacked directly by *Cl. botulinum* but only when a suitable H-acceptor is also present. Therefore, substitution of an hydroxyl group for a H-atom on the  $\beta$ -carbon of alanine produces a compound that may be employed singly as a source of energy by *Cl. botulinum*. These above results show that *Cl. botulinum* may obtain a portion of its energy requirements by direct utilization of amino acids such as serine as well as through coupled reactions between pairs of different amino acids (Stickland reaction) as previously described.

## 11273 P

### Nasopharyngeal Cultures in Pertussis \*

WILLIAM L. BRADFORD AND BETTY SLAVIN

*From the Departments of Pediatrics and Bacteriology, Rochester School of Medicine and Dentistry, Strong Memorial Hospital, and Rochester Municipal Hospital*

The generally accepted method for the bacteriological diagnosis of pertussis is the cough-plate culture, originally described by Chievitz and Meyer,<sup>1</sup> or some modification of their procedure. The percentage of positive cultures obtained by this technic varies considerably in the hands of different workers and under various conditions.<sup>2</sup>

We have found the cough-plate method satisfactory for older children, but in infants we have obtained positive cultures in only

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\* Supported by a grant from the Committee on Therapeutic Research of the American Medical Association and the Fluid Research Fund of the University of Rochester School of Medicine and Dentistry.

<sup>1</sup> Chievitz, J., and Meyer, A., *Ann. de l'Inst. Pasteur*, 1916, 30, 503.

<sup>2</sup> Sauer, L. W., *J. Ped.*, 1934, 5, 246.

about 25%, even when the cultures were made during the catarrhal period of the disease. This low incidence seems to be due to the fact that the young infant does not cough as vigorously as does the older child, thereby resulting in an inadequate inoculation of the medium.

During the course of a comparative study of various technics for making cultures, we were impressed by the favorable results obtained from culturing the nasopharynx. Recent reports<sup>3, 4, 5</sup> have described the advantages of the nasopharyngeal culture in the bacteriological diagnosis of pneumonia. This present report describes the results obtained by making simultaneous nasopharyngeal, throat and cough-plate cultures in a series of 25 consecutive cases of pertussis observed in the pediatric clinic during the past 4 months. The procedure follows:

The medium was prepared as previously described<sup>6</sup> and poured into ordinary Petri dishes. Medium more than 3 days old was discarded. The cough-plate cultures were made by exposing the medium during a paroxysm at a point about 6 inches in front of the patient's mouth. The nasopharyngeal cultures were taken by passing a sterile swab, consisting of a small bit of cotton tightly wrapped about the end of a thin, flexible copper wire, through the nose until it touched the posterior wall of the pharynx. Throat cultures were taken by means of a similar swab.

The results obtained in the present comparative study are shown in Table I.

Cultures were made on 25 cases, 17 of which were but 2 years of age or less. In this group, it is apparent that the results obtained by nasopharyngeal culture were distinctly superior to those by the throat culture or by the cough-plate method. In the 25 primary cultures, 17 were positive. Of this group, the nasal culture was positive in 14 instances, as compared to 8 positive cough-plates and to 4 positive throat cultures. Of the entire group of 40 cultures, there were 22 positive nasal, 10 positive cough-plates and 5 positive throat cultures. In only 3 cases were the cough-plate cultures positive when negative nasopharyngeal cultures were obtained. These patients were 4, 6, and 7 years of age, respectively, and were presumably able to cough vigorously.

<sup>3</sup> Hodes, H. L., Stifter, W. C., Jr., Walker, E., McCarly, M., and Shirlev, R. G., *J. Ped.*, 1939, 14, 417.

<sup>4</sup> Poole, F. D., and Fousek, M. D., *J. A. M. A.*, 1939, 113, 1854.

<sup>5</sup> Auger, W. J., *J. Ped.*, 1939, 15, 640.

<sup>6</sup> Bradford, W. L., and Slavin, B., *J. Clin. Investigation*, 1937, 16, 825.

TABLE I.  
A Comparison of Culture Methods in Pertussis

Patient	Age	Wk of disease	Cough plate	Throat	Naso pharynx	Patient	Age	Wk of disease	Cough plate	Throat	Naso pharynx	Patient	Age	Wk of disease	Cough plate	Throat	Naso pharynx
We	8 wk	2	0	0	+	Be	2 yr	2	0	0	+	Bi	9 mo	1	0	0	0
		4	0	0	+			4	0	0	0			2	+	0	+
Ho	3 mo	6	0	0	0			8	0	0	0			4	0	0	0
		4	+	+	+	Wa	0 wk	2	0	0	0	Sn	5 mo	3	0	0	+
Al	1 yr	6	0	0	+			3	0	0	0	O'K	3 yr	2	0	0	0
		6	0	0	+			4	0	0	0	O'K	6 "	2	+	0	0
DeL	3 mo	8	0	0	+	Sp	3 mo	5	0	0	0	Le	6 "	4	0	0	0
		5	0	0	0			7	+	+	+	Co	4 "	5	+	0	0
		6	0	0	0			10	0	0	0			8	0	0	0
Co	2 yr	1	0	0	+	McK	2 yr	2	0	0	+	Lo	7 "	4	+	0	0
Bu	2 "	4	+	0	+	Bo	2 "	3	0	0	+	Lo	8 "	2	0	0	+
		6	0	0	+	Ko	4 mo	2	0	0	+	Hn	4 "	4	0	0	0
Sm	7 wk	3	0	0	0	Hu	2 yr	1	+	+	+	Ba	8 mo	1	+	0	+
Ar	3 yr	2	+	0	+												

Of a total of 25 positive cultures, there were 23 nasal, 5 throat, and 10 cough plate  
Of the 25 primary cultures, 17 were positive, 14 nasal, 4 throat and 8 cough plate

In addition to the higher percentage of positive nasopharyngeal cultures obtained, the difference in the number of colonies which developed on the plates was striking. In repeated instances, there were only a few colonies of *Hemophilus pertussis* on a cough-plate or throat-plate culture, while the nasal culture revealed numerous colonies. Sometimes the growth was practically pure. The nasal culture often showed but a minimal growth of secondary invading organisms, while a heavy growth, on the other hand, usually occurred on the media inoculated from the throat. We believe that this difference in the occurrence of secondary invading organisms largely explains the difference in the percentage of positive cultures of *Hemophilus pertussis* isolated from the two sources. It may be, however, that the pertussis bacillus occurs in greater numbers in the nasopharynx during the disease and remains there for a longer period of time during convalescence. Further study will be necessary to decide this point.

A comparative study of the cough-plate, throat and nasopharyngeal cultures in pertussis indicates that the nasopharyngeal method is definitely the best, particularly for use in infants.

## 11274

# Anticatalase Activity of Sulfanilamide and Related Compounds V. Bacteriostatic Activity of some Sulfonhydroxamides

EDNA R. MAIN, LAWRENCE E. SHINN AND RALPH R. MELLON

From the Western Pennsylvania Hospital Institute of Pathology, Pittsburgh, Pa

This is a report of a study in which the ability of sulfanilamide to produce catalase inhibition is compared with that exhibited by acyl aminobenzenesulfonhydroxamides. Since the latter compounds contain a hydroxylamino group, they are analogous in structure to the intermediate assumed to be responsible for the anti-catalase activity of sulfanilamide in bacterial cultures<sup>1 2 3</sup>. Possibly because of the presence of this group, the sulfonhydroxamides are capable of exerting a more rapid and more intense bacteriostatic effect than sulfanilamide.

<sup>1</sup> Locke, A., Main, E. R., and Mellon, R. R., *Science*, 1939, **88**, 620

<sup>2</sup> Main, E. R., Shinn, L. E., and Mellon, P. R., *PROC. SOC. EXP. BIOL. AND MED.*, 1939, **37**, 272

<sup>3</sup> Shinn, L. E., Main, E. R., and Mellon, R. R., *PROC. SOC. EXP. BIOL. AND MED.*, 1939, **37**, 591



*Bacteriostatic Action* Fig 1 compares the speed and intensity of the bacteriostatic action of sulfanilamide and caproylaminobenzene-sulfonhydroxamide, the latter being chosen as typically representative of the group. Cultures, made by inoculating 10 cc quantities of veal infusion broth, contained in 50 cc Erlenmeyer flasks, with 0.0001 cc of a highly virulent, 18-hr broth culture of Type I pneumococcus, were incubated at 39.5°C. After 1.5 hrs, plate counts were made and the compounds added as freshly prepared solutions in 60% alcohol, the concentrations of which were so adjusted that an addition of 0.1 cc would give the desired final concentrations. The same volume of 60% alcohol was added to control cultures. Incubation was continued and samples were withdrawn for plate counts 0.75, 1.75, and 3.5 hrs after addition of the drugs.

The rate of generation was estimated from the counts by the formula

$$\frac{\text{count at the end of the observed interval}}{\text{count at the beginning of the interval}} = 2^n$$

The degree of retardation in rate of multiplication was obtained as the difference between the rate of generation in the control culture and in the culture containing the added drugs.

#### Example

Count 1.75 hrs after addition of compound = 1220

Count 3.5 hrs later = 6100

6100

1220 = 5.0 and therefore represents a 5.0 fold increase in population.

From the equation,  $5.0 = 2^n$ ,  $n = 2.3$  (total number of generations produced)

Number of generations produced per hr =  $2.3/1.75 = 1.3$

Since the number of generations per hr in control cultures for the corresponding time interval was 1.8, the difference, 0.5, represents the inhibition in rate of multiplication.

The results show a distinct difference in rate and intensity of bacteriostatic action of the two compounds. In the cultures containing sulfanilamide (shaded columns, Fig 1), a slight stimulation in rate of multiplication was observed at the end of the first interval. This was followed by inhibition which continued to increase during the remainder of the observation period. Inhibition by the sulfonhydroxamide (solid columns) was detectable by the end of the first interval and rapidly increased in magnitude, presumably as a result of increasing peroxide accumulation. By the end of the second period of observation, the inhibition was 2.5 times greater than that of sulfanilamide although the concentration was less than one-third as great.

*Peroxide Accumulation and Growth Inhibition* Table I indicates

the comparative capacities of sulfanilamide and the sulfonhydroxamides for producing peroxide accumulation and growth inhibition. The ratios of peroxide concentration to amount of growth were determined under the experimental conditions previously described.<sup>4</sup> Alcoholic solutions of the drugs were used as in the experiments described above, but were added before inoculation. The inoculum, 0.1 cc of an 18-hr culture, was larger than that used in the above experiments in order that growth would be sufficiently large to be measured turbidimetrically from the fourth to the seventh hour of growth. Only the ratios found at the seventh hour are reported in the table. Under the conditions of the experiment, peroxide was usually detectable by the peroxidase test\* by the third hour.

In cultures containing 0.00014 to 0.00020 *M* caproylamino benzene-sulfonhydroxamide, the peroxide concentration per unit of growth was from 2 to 4 times greater than in control cultures. With a 0.00052 *M* concentration there was no visible growth during the 7 hours. In cultures containing 0.00052 *M* sulfanilamide, peroxide concentration per unit of growth was, on the average, 4 times greater than that of the control. In the presence of 0.00052 *M* acetamido-benzenesulfonhydroxamide the ratio was from 2 to 3 times greater and therefore this compound was only slightly less effective than sulfanilamide in the same concentration. Toluene- and benzene-sulfonhydroxamides were approximately equal in effect to the acetamido compound.

*Anti-Catalase Activity* The hydroxamides listed in Table I showed anti-catalase activity equal to or greater than that of irradiated sulfanilamide. The activity was not, however, constant. Upon standing in solution the sulfonhydroxamides undergo a change, shown by the development of a yellow color and an altered reaction with *o*-toluidine.

<sup>4</sup> Main, E. R., Shinn, L. E., and Mellon, R. R., *Proc Soc Exp Biol and Med*, 1939, **42**, 115.

\* A slight modification of the method previously described (Main and Shinn, *J Biol Chem*, 1939, **128**, 417) was used. An aqueous extract of dried horseradish was used in place of the potato extract as the source of peroxidase, as suggested by Fuller and Maxted (*Brit J Exp Path*, 1929, **20**, 177). Determinations can be made over the range, 2 to 20% per cc, while with potato extract, the range is 6 to 30%.

It was necessary to use an additional blank in the determination of peroxide in the presence of sulfonhydroxamides, since, after standing at a pH of 7.0 or above for one half hour or more, solutions of the sulfonhydroxamides give a yellow to orange color upon addition of the *o*-toluidine reagent. To make color comparisons with the peroxide standards, a blank consisting of sterile broth which contains the same concentration of compound as the culture is placed back of the standard tube in the comparator.

TABLE I

Effect of Sulfonhydroxamides on Growth and Peroxide Accumulation in Broth Cultures of Type I Pneumococcus

Ten cc quantities of veal infusion broth containing 0.2% glucose and the compounds in the concentrations given below were inoculated with 0.1 cc of an 18 hr broth culture of Type I pneumococcus. Samples were removed at intervals for determination of growth and peroxide content. The ratios, A and B, were calculated from determinations made after 7 hr growth.

Compound added	Millimols per lit	H <sub>2</sub> O <sub>2</sub> concentration per unit of growth† in		Ratio, B/A
		A Control cultures	B Cultures contg compds	
4-n-Caproylamino benzenesulfon hydroxamide*	0.52	0.3	—§	—
	20	3	1.1	3.7
	20	4	8	2.0
	17	4	8	2.0
	14	2	5	2.5
	14	4	7	1.8
4-Acetamidobenzenesulfon hydroxamide	52	.2	6	3.0
	52	.2	4	2.0
	52	3	1.0	3.3
p-Toluenesulfonhydroxamide	52	2	5	2.5
	52	.2	.5	2.5
	52	3	4	1.3
Benzenesulfonhydroxamide	52	.2	6	3.0
	.52	3	7	2.3
	.20	3	7	2.3
	17	4	5	1.3
Sulfanilamide	52	3	1.2	4.0

\* The sulfonhydroxamides were furnished to us through the courtesy of Sharp and Dohme, Inc.

† Stock solutions of the compounds in 60% alcohol were prepared in such concentrations that 0.1 cc contained the amount needed to give the final concentration. The control cultures contained 0.1 cc alcohol.

‡ Growth was estimated by comparisons with BaSO<sub>4</sub> standards which had been calibrated against a culture arbitrarily selected to represent 100% growth. The growth units are therefore percentages relative to the standard culture. H<sub>2</sub>O<sub>2</sub> concentration is expressed in micrograms per cc.

§ There was no visible growth in cultures containing this concentration.

|| This figure represents the average of all determinations made with sulfanilamide-containing cultures, which were included regularly as controls.

**Discussion** Sulfonamide derivatives studied heretofore have contained a para amino group which is convertible into a hydroxylamino group, giving the compound anti-catalase properties. In bacterial cultures, these compounds check the action of catalase and thereby cause increased accumulation of peroxide. The amount of active intermediate present may be very small, its effect depending on its continuous release in the vicinity of the cell. A compound which contains a preformed hydroxylamino group should act more rapidly

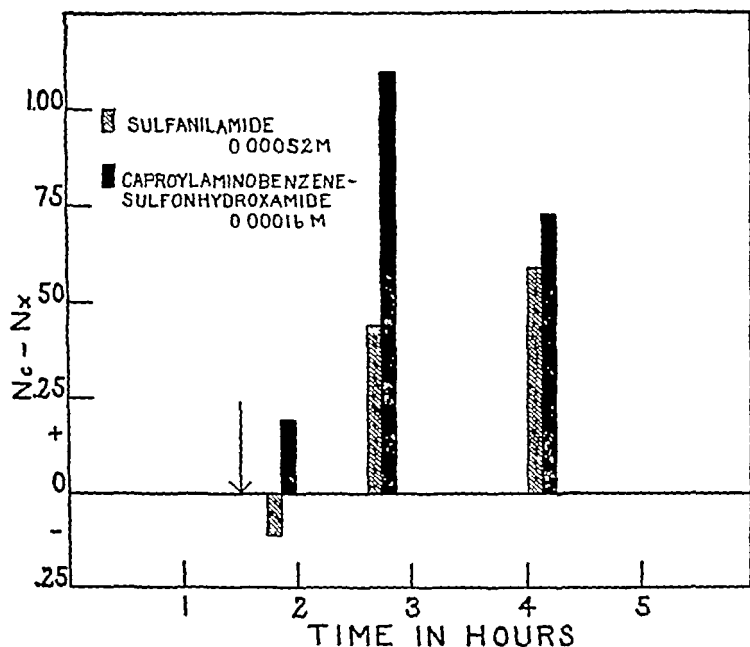


Fig 1

Comparison of the rates of development of bacteriostasis in the presence of sulfanilamide and 4-*n*-caproylaminobenzenesulfonhydroxamide

$N_c$  is the number of generations produced per hour in the control cultures and  $N_x$  the number produced in the cultures containing compound added. The additions were made at the time indicated by the arrow. Each column represents the average of results from 10 experiments.

and because of the presence of active substance in high concentration, should have a more intense effect. The experiments reported here indicate that this is the case with the acyl aminobenzenesulfonhydroxamides, compounds of the structure,  $RNH-C_6H_4-SO_2NHOH$ , where R is the caproyl, valeryl, heptanoyl or acetyl group. When compared mole for mole, caproylaminobenzenesulfonhydroxamide is a much stronger bacteriostatic agent than sulfanilamide. Bacteriostatic activity is demonstrable almost immediately and increases rapidly while peroxide is accumulating. Since the toluene and benzenesulfonhydroxamides compounds which contain no para amino group, caused marked stasis and peroxide accumulation, the activity of the acyl compounds *in vitro* is apparently not due to a deacylated amino group.

The results given here apply only to stasis in broth cultures. The concentration of the hydroxamide required to produce the same amount of stasis in blood culture is appreciably greater. Results of *in*

TABLE I

Effect of Sulfonhydroxamides on Growth and Peroxide Accumulation in Broth Cultures of Type I Pneumococcus

Ten cc quantities of veal infusion broth containing 0.2% glucose and the compounds in the concentrations given below were inoculated with 0.1 cc of an 18 hr broth culture of Type I pneumococcus. Samples were removed at intervals for determination of growth and peroxide content. The ratios, A and B, were calculated from determinations made after 7 hr growth

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		A Control cultures	B Cultures contg compds	
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	20	3	1.1	3.7
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	14	4	7	1.8
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	52	2	4	2.0
	52	3	1.0	3.3
<i>p</i> -Toluenesulfonhydroxamide	52	2	5	2.5
	52	2	.5	2.5
	52	3	4	1.3
Benzenesulfonhydroxamide	52	2	6	3.0
	52	3	7	2.3
	.20	.3	7	2.3
	17	4	5	1.3
Sulfanilamide	52	3	1.2	4.0

\* The sulfonhydroxamides were furnished to us through the courtesy of Sharp and Dohme, Inc.

† Stock solutions of the compounds in 60% alcohol were prepared in such concentrations that 0.1 cc contained the amount needed to give the final concentration. The control cultures contained 0.1 cc alcohol.

‡ Growth was estimated by comparisons with BaSO<sub>4</sub> standards which had been calibrated against a culture arbitrarily selected to represent 100% growth. The growth units are therefore percentages relative to the standard culture. H<sub>2</sub>O<sub>2</sub> concentration is expressed in micrograms per cc.

§ There was no visible growth in cultures containing this concentration.

|| This figure represents the average of all determinations made with sulfanilamide-containing cultures, which were included regularly as controls.

**Discussion** Sulfonamide derivatives studied heretofore have contained a para amino group which is convertible into a hydroxylamino group, giving the compound anti-catalase properties. In bacterial cultures, these compounds check the action of catalase and thereby cause increased accumulation of peroxide. The amount of active intermediate present may be very small, its effect depending on its continuous release in the vicinity of the cell. A compound which contains a preformed hydroxylamino group should act more rapidly.

TABLE I  
Gonadotropic Potency of Male and Female Castrates' Pituitary Before and After Tryptic Digestion

Group	Type of pituitary*	Total dose, mg	No of immature rats	Mean and S E of paired ovarian wt, mg	Mean and S E of uterine wt, mg	Groups compared	Pl	
							ovaries	uterus
1	F	50	9	69.76 ± 4.90	82.10 ± 3.61	1 2	0	17
2	F, T	100	12	28.73 ± 1.42	63.77 ± 10.66	1 3	66	88
3	M	50	10	66.05 ± 4.74	82.89 ± 4.45	2 4	65	66
4	M, T	100	10	22.81 ± 1.33	40.35 ± 1.67	3 4	0	0
5	FSH	10	10	52.27 ± 8.70	90.70 ± 0.08	5 6	69	73
6	FSH, T	10	11	47.55 ± 7.00	81.88 ± 9.90			

\* F—Female castrate, M—Male castrate, FSH—Follicle stimulating hormone, T—Trypsin digested

† Probability that random sampling would give as great a difference according to Fisher's method<sup>1</sup> for testing significance of difference of means

<sup>1</sup> Fisher, R. A., *Statistical Methods for Research Workers*, Oliver and Boyd, Edinburgh, 1930

*in vivo* experimentation<sup>5</sup> indicate that when given daily in doses of 1 mg per g for 6 days, the acyl aminobenzenesulfonhydroxamides and sulfanilamide possess approximately the same therapeutic activity against pneumococcus infection in mice

*Summary* The acyl aminobenzenesulfonhydroxamides have strong anti-catalase activity and, when present in broth cultures of the pneumococcus, cause inhibition of growth associated with increased accumulation of hydrogen peroxide. When the caproyl compound is added to growing cultures, inhibition of growth is detectable almost immediately but reaches a maximum only after time for accumulation of peroxide has elapsed. Inhibition by sulfanilamide, on the other hand, is detectable somewhat later and approaches a maximum more slowly

## 11275

## Gonadotropic Potency of Gonadectomized Rats' Pituitary after Tryptic Digestion

R C LI

*From the Department of Pharmacology, Peiping Union Medical College*

The evaluation of the amount of the follicle-stimulating and luteinizing hormones of the pituitary of normal as well as gonadectomized animals has interested a number of investigators. The recent reports of McShan and Meyer<sup>1</sup> and Chen and van Dyke<sup>2</sup> on tryptic digestion of pituitary extract appear to afford a convenient means of estimating the relative amounts of these fractions, as the luteinizing activity is largely destroyed by trypsin to which the follicle-stimulating activity is resistant. In the following experiments the gonadotropic activity of castrate rats' pituitary following tryptic digestion was compared with that of the castrates' pituitary not subjected to such treatment.

The donors of the pituitary consisted of 24 male and 38 female albino rats which were gonadectomized at the age of 1-3 months. Three to 6 months (usually 3 months) after gonadectomy, the animals were sacrificed. The anterior pituitary was obtained, weighed, and ground up fresh in an appropriate quantity of 0.02%

<sup>5</sup> Cooper, F. B., Gross, P., and Lewis, M., *Proc. Soc. Exp. Biol. and Med.*, 1940, **43**, 491.

<sup>1</sup> McShan, W. H., and Meyer, R. K., *J. Biol. Chem.*, 1938, **126**, 361.

<sup>2</sup> Chen, G., and van Dyke, H. B., *Proc. Soc. Exp. Biol. and Med.*, 1939, **40**, 172.

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PEIPING

Peiping Union Medical College	February	7, 1940
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11276 P

Effect of Meat Extract on Fatty Infiltration of Liver in Depan-  
creatized and Duct-Ligated Dogs

ELAINE P RALLI AND SAUL H RUBIN

*From the Laboratories of the Department of Medicine, New York University  
College of Medicine*

Since Fisher<sup>1</sup> and Allan, *et al*,<sup>2</sup> first reported fatty infiltration of the livers in depancreatized dogs maintained on insulin, the observation has been confirmed repeatedly.<sup>3</sup> Recently, it has been shown in

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<sup>1</sup> Fisher, N F, *Am J Physiol*, 1924, 67, 634.

<sup>2</sup> Allan, F N, Bowie, D J, MacLeod, J J R, and Robinson, W L, *Brit J Exp Path*, 1924, 5, 75

<sup>3</sup> Best, C H, and Ridout, J H, in Luck, J M., *Ann Rev Biochem*, 1939, 8, 349

this<sup>4</sup> and other<sup>5</sup> laboratories that ligation of the external pancreatic ducts results in similar fatty changes in the liver. In all of these studies the diet fed to the dogs consisted largely of raw whole meat and glucose.

In the experiments reported here a dried meat powder preparation,\* from which the extractives had been removed, was substituted for the whole meat. The diet contained meat powder, cracker meal, milk powder, salts, brewer's yeast, bone ash, cod liver and corn oil. As shown in Table I, little or no fatty infiltration of the liver occurred in 6 depancreatized and in 3 duct-ligated dogs which were fed the diet for periods varying from 6.5 to 19 weeks after operation. The fatty acid content of the whole livers, determined as previously described,<sup>7</sup> averaged 6.2% with a range of 3.12 to 10.5% (Table I). When the meat powder diet was supplemented with 15 ml of a concentrated meat extract\* (the combination of meat powder plus extract being equivalent to about 250 g of raw lean beef) the livers of

TABLE I  
Liver Lipids of Depancreatized and Duct Ligated Dogs Fed Meat Powder or Meat Powder Plus Meat Extract

Dog No	Condition of animal*	Meat supplement†		Time after operation, wks	Body wt		Liver		
		Powder, g/day	Extr, ml/day		Initial, kg	Final, kg	Wt, g	Fatty acids, %	Total cholesterol, %
180	D	85	0	6.5	10.3	8.0	358	3.1	27
176	D	35	0	12	8.0	6.4	232	10.4	33
192	D	35	0	12	15.8	10.0	470	9.0	23
196	D	50	0	11	13.0	12.3		3.4	22
170	D	50	0	13	13.3	12.8	416	3.2	23
198	D	75	0	19	10.3	11.3	403	4.4	24
147	L	35	0	14	13.0	7.4	212	10.5	35
193	L	35	0	13.5	11.8	10.0	273	3.7	29
189	L	50	0	12	11.8	9.8	267	8.2	32
216	D	50	15	5	9.8	6.8	377	19.5	25
214	D	75	15	7	9.8	7.0	456	18.3	29
203	L	50	15	9	9.3	6.8	294	19.8	23

\* D = depancreatized, L = duct-ligated dog  
† 60 g meat powder + 15 ml extract  $\approx$  250 g whole meat.

<sup>4</sup> Balli, E. P., Rubin, S. H., and Present, C. H., *Am J Physiol*, 1938, **122**, 43

<sup>5</sup> Person, E. C., Jr., and Glenn, F., *Arch Surg*, 1939, **39**, 530

<sup>6</sup> Montgomery, M. L., Entenman, C., and Chaikoff, I. L., *J Biol Chem*, 1939, **128**, 387

\* Dried meat powder and concentrated meat extract were purchased from the Valentine Meat Juice Company, Richmond, Va. The meat extract is a hot water extract of lean beef. It contains 4.5% proteose and peptone after it has been concentrated. This concentrated extract is what was used. Practically all of the protein and fat of the beef is in the powder.

<sup>7</sup> Rubin, S. H., Present, C. H., and Balli, E. P., *J Biol Chem*, 1937, **121**, 19

one duct-ligated and 2 depancreatized dogs contained 18.3 to 19.8% fatty acids after 5 to 9 weeks (Table I). It is therefore concluded that the fatty liver of these dogs is due in large measure to some substance or substances present in the extractive fraction of meat.

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### Analysis of the Bile of the Capuchin Monkey

FRANCES F. BECK (Introduced by John C. Krantz, Jr.)

From the Department of Pharmacology, School of Medicine, University of Maryland

Studies in this laboratory in recent years have been concerned with the hydrogen-ion concentration of the bile of the guinea pig<sup>1</sup> and dog<sup>2</sup> with respect to the dissolution of human gall stones. Having available a series of Capuchin monkeys (*Cebus fatuellus*) upon which metabolism studies had been conducted, it was deemed of interest to study the bile of this species of monkey. The animals were narcotized with sodium amytal, the bile was drawn from the gall bladder without exposure to air. The hydrogen-ion concentration was determined by means of a glass electrode at 25° and an analysis of the principal constituents was carried out according to the Douglas-Sauermann<sup>3</sup> method. The results on 10 animals are shown in Table I.

The average pH of the bile and the percentage of various constituents present in the Capuchin monkey are similar to these data on the gall-bladder bile of dogs.<sup>2</sup> Also as with the bile of the dog it was observed that the correlation between a high pH and high total solids content is great.

TABLE I  
Constituents of Monkey Gall Bladder Bile

	pH	Total solids, %	Mucin, %	Ash, %	Alkalinity of ash as % Na <sub>2</sub> CO <sub>3</sub> in bile	Lipoids, %	Bile acids as cholic and desoxycholic, %
Aver	6.3	20.8	0.81	1.65	0.65	7.95	6.05
Low	5.6	10.3	0.46	1.04	0.26	3.81	4.30
High	6.9	28.8	1.15	2.06	0.99	11.30	10.00

<sup>1</sup> Krantz, J. C., Jr., Feldman, M., Morrison, S., and Carr, C. J., *Proc. Soc. Exp. Biol. and Med.*, 1936, **35**, 48.

<sup>2</sup> Beck, F. F., Krantz, J. C., Jr., Feldman, M., and Carr, C. J., *Proc. Soc. Exp. Biol. and Med.*, 1937, **37**, 357.

<sup>3</sup> Douglas-Sauermann, A. G., *Z. Physiol. Chem.*, 1935, **231**, 92.

## Nature of the Urinary Androgens of Castrate Men

WALTER HOSKINS\* AND BRUCE WEBSTER

*From the Barbara Henry Research Laboratory of the New York Hospital and Department of Medicine, Cornell University Medical College, New York City*

A number of investigators have reported small amounts of urinary androgens in human male castrates<sup>1-5</sup> but the nature of the active material has not been established. A comparison of this androgenic material with that from the urine of normal men is of interest in view of the increasing use of urinary androgen values as measures of testis activity

The present report describes the measurement of the androgens in the urine of male castrates and the fractionation of this material with digitonin in an effort to characterize the androgens present

A complete urine collection was made from two male surgical castrates for 9 and 7 days respectively and the urines pooled. One subject had been castrated 10 years previously at the age of 37, the other 35 years before at the age of 26. The urine was kept in the refrigerator without added preservative and used within 3 days after its collection. Acid hydrolysis, benzene extraction and separation into androgenic and estrogenic fractions were carried out according to the methods of Gallagher, *et al*.<sup>6</sup> The androgenic fraction was taken up in 60% alcohol and assayed by inunction on the capon comb in parallel with pure androsterone as a standard.<sup>†</sup> Assays on groups of 4 to 6 capons showed an androgen excretion of 6 international units per day.

One-half of the androgenic fraction, the equivalent of 8 days' excretion, was treated with digitonin to separate any dehydroisoandrosterone (or other sterols having an hydroxyl group at position 3 in the beta configuration) as the insoluble digitonide.<sup>7</sup> The

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\* General Education Board and Barbara Henry Research Fellow

1 Koch, F. C., *J. Urology*, 1936, **35**, 382

2 Chou, C. Y., and Wu, H., *Chinese J. Physiol.*, 1937, **11**, 429

3 Callow, B. K., *Proc. Roy. Soc. Med.*, 1938, **31**, 841.

4 Hansen, E. H., *Endokrinologie*, 1938, **21**, 9

5 McCullagh, E. P., *J. A. M. A.*, 1939, **112**, 1037

6 Gallagher, T. F., Peterson, D. H., Dorfman, R. L., Kenyon, A. T., and Koch, F. C., *J. Clin. Invest.*, 1937, **16**, 695

† We are grateful to the Ciba Corporation, Lafayette Park, New Jersey, for the androgens used and to Robert Kinloch for his assistance with the assays

7 Butenandt, A., Dannenbaum, H., Harnisch, G., and Kudzusz, H., *J. Physiol. Chem.*, 1935, **237**, 57

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- 1 Koeh, F. C., *J Urology*, 1936, **35**, 382
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- † We are grateful to the Ciba Corporation, Lafayette Park, New Jersey, for the androgens used and to Robert Kamloch for his assistance with the assays
- 7 Butenandt, A., Dannenbaum, H., Hanisch, G., and Kudzus, H., *Z Physiol Chem*, 1935, **287**, 57

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7 Butenandt, A., Dannenbaum, H., Hanisch, G., and Kudzus, H., *Z. Physiol. Chem.*, 1935, **237**, 57

TABLE I  
Androgen Content of Urine fractions of Normal and Castrate Men

TABLE 1 Digitonin treated urine extracts						Ratio A to D by wt
Androgen Content of Urine fractions of Normal and						
Untreated urine (IU/day)	Androsterone		Dehydroisandrosterone		% A ‡ by wt	% D § by wt
	γ/day	IU/day	γ/day	IU/day		
	2025 150	29 25 15	Obs	Corr	59 82	41 18
		1400 70	2000* 100*	0 25† 0 3†		
Correct for incomplete separation as measured on known solutions						
Correct for incomplete separation as measured on basis of activity ratios by the innunction assay method						

Normal  
Castrate

\* Observed dehydro values divided by 0.7 to correct for incomplete separation as measured on known solutions

† Gamma of dehydro converted to IU (100 gamma of androsterone equal to 3.20 gamma of dehydro)

‡ Gamma of androsterone

§ A equals androsterone

§ D equals dehydroisoandrosterone

measured on known solutions by the inunction assay method



digitonide was decomposed with pyridine and the dehydroisoandrosterone and androsterone fractions assayed in parallel with the pure androgens. An extract of normal male urine was treated similarly. Control experiments with known solutions of dehydroisoandrosterone gave only a 70% recovery with digitonin treatment so the values observed for the urine fractions have been corrected for this loss. The results are shown in Table I.

The value found for the total androgen excretion of male castrates, 6 IU per day, agrees with the values previously reported, most of which are between 1 and 10 IU per day although Callow reports one case with 30-39 IU per day. The ratio of androsterone to dehydroisoandrosterone in male castrate urine, 4:1, contrasts with the approximately 1:1 ratio previously reported for normal male urine<sup>7,8</sup> and the 3:2 ratio found here. Attempts to isolate androgens from these fractions were unsuccessful. In the absence of actual identification of androsterone or dehydroisoandrosterone considerable caution must be used in interpreting these figures. On the other hand, in view of the presence of these two androgens in the urine of normal men and women and female castrates<sup>7,9,10</sup> it seems reasonable to assume that these two compounds make up the greater part of the biologically active material. (An inactive epimer of androsterone,  $\alpha$ -3-hydroxyetiocholanone-17, has been isolated in amounts equal to androsterone from the urine of normal men and of female castrates<sup>7,10</sup>.) Hansen<sup>4</sup> suggests that the androgenic material in male castrate urine resembles  $\Delta$ -4-androstenedione in its biological action. He did not consider the possible presence of dehydroisoandrosterone and the properties he mentions can probably be explained as due to the combined actions of dehydroisoandrosterone and androsterone rather than to androstenedione.

It is apparent from Table I that the sum of the androgenic activities in the androsterone and dehydroisoandrosterone fractions is about 20% less than the total androgens as determined on the unfractionated urine. This difference is due to errors arising from the manipulations involved, the biological assay, the rather arbitrary correction factor applied to the dehydroisoandrosterone values and the activity ratio used in converting gamma of dehydroisoandrosterone to gamma of androsterone (IU). The existence of this difference and the nature of the methods used make it inadvisable to theorize from the present data but at the same time these ratios have interesting implications.

<sup>8</sup> Dingemans, E., and Laqueur, E., *Biochem. J.*, 1938, **32**, 651.

<sup>9</sup> Callow, N. H., and Callow, R. K., *Biochem. J.*, 1939, **33**, 931.

<sup>10</sup> Hirschmann, H., *J. Biol. Chem.*, 1939, **130**, 421.

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<sup>9</sup> Callow, N. H., and Callow, R. K., *Biochem. J.*, 1939, **33**, 931.  
<sup>10</sup> Hirschmann, H., *J. Biol. Chem.*, 1939, **130**, 421.

digitonide was decomposed with pyridine and the dehydroisoandrosterone and androsterone fractions assayed in parallel with the pure androgens. An extract of normal male urine was treated similarly. Control experiments with known solutions of dehydroisoandrosterone gave only a 70% recovery with digitonin treatment so the values observed for the urine fractions have been corrected for this loss. The results are shown in Table I.

The value found for the total androgen excretion of male castrates, 6 IU per day, agrees with the values previously reported, most of which are between 1 and 10 IU per day although Callow reports one case with 30-39 IU per day. The ratio of androsterone to dehydroisoandrosterone in male castrate urine, 4:1, contrasts with the approximately 1:1 ratio previously reported for normal male urine<sup>7, 8</sup> and the 3:2 ratio found here. Attempts to isolate androgens from these fractions were unsuccessful. In the absence of actual identification of androsterone or dehydroisoandrosterone considerable caution must be used in interpreting these figures. On the other hand, in view of the presence of these two androgens in the urine of normal men and women and female castrates<sup>7, 9, 10</sup> it seems reasonable to assume that these two compounds make up the greater part of the biologically active material. (An inactive epimer of androsterone, alpha-3-hydroxyetiocholanone-17, has been isolated in amounts equal to androsterone from the urine of normal men and of female castrates<sup>7, 10</sup>.) Hansen<sup>4</sup> suggests that the androgenic material in male castrate urine resembles  $\Delta$ -4-androstenedione in its biological action. He did not consider the possible presence of dehydroisoandrosterone and the properties he mentions can probably be explained as due to the combined actions of dehydroisoandrosterone and androsterone rather than to androstenedione.

It is apparent from Table I that the sum of the androgenic activities in the androsterone and dehydroisoandrosterone fractions is about 20% less than the total androgens as determined on the unfractionated urine. This difference is due to errors arising from the manipulations involved, the biological assay, the rather arbitrary correction factor applied to the dehydroisoandrosterone values and the activity ratio used in converting gamma of dehydroisoandrosterone to gamma of androsterone (IU). The existence of this difference and the nature of the methods used make it inadvisable to theorize from the present data but at the same time these ratios have interesting implications.

<sup>8</sup> Dingemans, E., and Laqueur, E., *Biochem. J.*, 1938, **32**, 651.

<sup>9</sup> Callow, N. H., and Callow, R. K., *Biochem. J.*, 1939, **33**, 931.

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The isolation of steroids from the adrenal cortex closely resembling the common androgens, some having androgenic activity, and the high levels of urinary androgens in some cases of adrenal tumor seem to point to the adrenal as the source of the extragonadal urinary androgens. Callow<sup>3</sup> has discussed this possibility and in view of the isolation of large quantities of dehydroisoandrosterone from the urine of patients with adrenal tumors suggests that all the dehydroisoandrosterone and most of the other androgenic activity in normal male urine is derived from the adrenal and not the testis. With this suggestion in mind it is surprising to find that the male castrate urine contained a smaller rather than a larger proportion of dehydroisoandrosterone than normal urine. It is conceivable that the testis normally transforms a portion of the steroids elaborated by the adrenal cortex into the testis hormone which is subsequently changed by other tissues into suitable excretion forms, androsterone and its inactive epimer and dehydroisoandrosterone. A small part of the steroids appears to go through this or a similar series of reactions independently of the testis and can be found in the urine when the testis is absent as the same androgens but in slightly different proportions.

The measurement of the relative amounts of these two types of urinary androgens by digitonin fractionation is being carried out on the urine of female castrates, boys and girls, in an effort to extend the data.

*Summary* A pooled urine collection from 2 male castrates contained 6 I U per day of androgenic activity. Capon assay of the results of digitonin fractionation indicated an androsterone to dehydroisoandrosterone ratio of 4:1 compared with the approximately 1:1 ratio found in normal male urine. In the absence of the testis smaller amounts of the same or similar androgens are excreted in slightly different proportions.

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## Quantitative Studies on Relationship Between Estrogen and Mammary Gland Carcinoma in Strain C3H Mice

EDWARD L. BURNS AND JOHN R. SCHENKEN (Introduced by Kenneth L. Burdon)

*From the Department of Pathology and Bacteriology, School of Medicine, Louisiana State University, New Orleans*

The first experimental proof of a qualitative and quantitative relationship between the internal secretions of the ovary and carcinoma of the mammary gland in mice was presented by Leo Loeb<sup>1, 2</sup> and more recent investigations<sup>3</sup> have amply confirmed the qualitative phase of this relationship. The following experiments concern the quantitative aspects of the problem.

**Materials and Methods** The data were obtained from 206 strain C3H mice\* of which 122 were experimental and 84 were control animals. Their diet consisted of fresh Purina dog chow, to which lettuce was added at irregular intervals.

All the experimental animals were males and all were at least 5 months old when they died or were sacrificed. They were divided into 7 groups (Table I) and were injected with estradiol benzoate in a solution of sesame oil (Progynon-B) from the age of 2 weeks onward as follows:

Group I received 3000 rat units in 2 doses over a period of 3 days.

Groups II-VI inclusive received 100 rat units weekly for 4, 8, 12, 16, and 20 weeks respectively.

Group VII received 100 rat units weekly throughout the duration of life.

The control groups consisted of 38 untreated breeding male (Group VIII) and 46 untreated breeding female mice (Group IX).

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<sup>1</sup> Lathrop, A. E. C., and Loeb, Leo, *J. Cancer Res.*, 1916, **1**, 1.

<sup>2</sup> Loeb, Leo, *J. M. Research*, 1919, **40**, 477.

<sup>3</sup> Cori, C. F., *J. Exp. Med.*, 1927, **45**, 983; Murray, W. S., *J. Cancer Res.*, 1928, **12**, 18; Lacassagne, A., *Compt. rend. Acad. d. sc.*, 1932, **195**, 630; Bonser, G. M., *J. Path. and Bact.*, 1935, **41**, 33; Burrows, H., *Am. J. Cancer*, 1935, **24**, 613; Cramer, W., and Horning, E. S., *Lancet*, 1936, **1**, 247; Sontzkeff, V., Burns, E. L., Moskop, M., and Loeb, Leo, *Am. J. Cancer*, 1936, **27**, 229.

\* The parent animals were obtained in 1938 from the Roscoe B. Jackson Memorial Laboratory at Bar Harbor, Maine. In New Orleans the strain has been propagated only by brother to sister matings. A high percentage of spontaneous carcinoma of the mammary gland occurs among the breeding females of this strain.

TABLE I

TABLE I

Group	Experimental mice							Control mice	
	I	II	III	IV	V	VI	VII	VIII	IX
Incidence of Tumors in Relation to Dosage of Estrogen									
No of animals	20	11	20	14	19	19	19	18	40
No of tumors	0	0	3	1	11	9	10	0	33
Incidence tumors (%)	0	0	15.0	7.1	57.8	17.1	52.6	0	82.5
Total dosage rat units	3000	400	800	1200	1600	2000	Avg 3270	0	0
No of doses	2	4	8	12	16	20	Avg 32.7	0	0
Mode of dosage	Alternate days at age 2 weeks weekly weekly weekly weekly weekly weekly weekly weekly weekly								
Incidence of Tumors in Relation to Age in Months									
Age range	0-30	8-86	5-13	0-20	0-90	5-43	5-66	0-90	0-93
Avg age at which injections were ended	19.83	18.80	10.10	18.30	13.36	15.53	12.46	18.60	18.86
Avg interval between last injection and death	0.53	1.06	2.10	3.00	4.04	1.94	7.90	—	—
With tumors	—	—	4.83	7.10	5.93	4.20	0	—	—
Without tumors	13.50	10.00	7.73	7.75	7.10	5.53	0	—	—
Entire group	13.50	10.00	7.30	7.33	4.76	4.86	0	—	—
Avg age at death	—	—	6.00	6.00	9.86	9.06	8.60	—	13.00
With tumors	14.03	11.06	9.83	10.00	7.40	11.56	7.08	14.03	9.86
Without tumors	14.03	11.06	9.10	10.33	8.80	9.80	7.90	14.03	13.03
Entire group	—	—	—	—	—	—	—	—	—



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Control mice

STROGEN AND MAMMARY

TABLE I

Experimental mice

Control mice

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Group

Incidence of Tumors in Relation to Dosage of Estrogen

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all of which were over 6 months of age at the time of necropsy (Table I)

It is important to note that after the specified course of injections the mice in Groups I-VI inclusive received no further treatment.

The presence or absence of tumors was determined by gross inspection at necropsy, a sufficient number of tumors having been examined microscopically to establish the accuracy of gross diagnosis. Small masses of questionable identity were not regarded as neoplasms.

*Results* No tumors developed in the animals of Group I or Group II. A low incidence was observed in the animals of Groups III and IV, and the tumors developed at an earlier age in the mice of these groups than in the mice of any other experimental group. Moreover, all 3 animals which developed tumors in Group III were from the same litter. A relatively high and essentially equal percentage of tumors developed in mice of Groups V, VI and VII and the neoplasms occurred at approximately the same average age in each group. The female controls (Group IX) developed a higher percentage of tumors than any of the experimental groups, and the tumors appeared at an older age. No tumors were noted in the male controls (Group VIII). The complete data are shown in Table I.

*Summary* 1 Beginning at the age of 2 weeks, 122 non-breeding male strain C3H mice were treated with estrogen for varying periods of time. At the end of the specified course of injections the mice received no further treatment until they died or were sacrificed. 2 Four weekly doses of 100 rat units of estrogen produced no mammary gland carcinomas, 8 and 12 weekly doses of 100 rat units produced a low incidence of these tumors. 3 After 16 weekly doses of 100 rat units of estrogen had been given a relatively high percentage of carcinoma of the mammary gland developed, the administration of 20 weekly doses or an average of 32.7 weekly doses of 100 rat units did not raise the incidence of tumors above this level. 4 Three thousand rat units of estrogen administered over a period of 3 days at the age of 2 weeks produced no tumors.

## Effect of Inhaled Carbon Dioxide in Rheumatoid (Atrophic) Arthritis

ARTHUR LOCKE AND MORRIS A. COHEN

*From the Institute of Pathology and the Department of Orthopedics of the Western Pennsylvania Hospital, Pittsburgh*

A part of the limitation to movement in rheumatoid (atrophic) arthritis is due to muscle spasm and pain, not to limiting changes in structure. It can be lessened through administration of carbon dioxide and returned through acceleration of carbon dioxide loss (over-breathing) to a degree suggesting relationship to the phenomenon of hyperventilation tetany.

The 10 subjects of the experiment summarized in Table I were given inhalations of 10 to 15% carbon dioxide in oxygen for 2 to 5 minutes, from a Tissot spirometer, following determination of the degree of limitation to motion existing prior to inhalation. All responded to the inhalation with the immediate, transient, measurable and significant decrease in limitation indicated. A subjective impression of decrease in pain was reported, following first inhalation of  $\text{CO}_2$ , by all of the group but one. It could be corroborated by observation, before and after inhalation, of the amount of passive flexion or pressure required to elicit the painful sensation in question, in only 6. The effect on pain, like the effect on limitation of motion, was not observed following inhalation of oxygen unmixed with  $\text{CO}_2$  but was more certainly elicited by mixtures of  $\text{CO}_2$  with oxygen than by mixtures of  $\text{CO}_2$  with air. Both effects persisted longest in those of the subjects making greatest effort to guard against over-breathing through self-control, stimulation of  $\text{CO}_2$ -production through persevering mild, non-painful exercise, and precaution against chilling. Cumulative effect, following repeated inhalation, was obtained only up to the point of limitation by factors other than muscle spasm and pain.

Carbon dioxide was less certain and definite in effect on the conditions grouped in Table II.

Administration of 10 to 20%  $\text{CO}_2$  in oxygen to rabbits was followed by the transient decrease in responsiveness to the stimulus of chilling<sup>1</sup> indicated in Fig. 1 and the associated<sup>2</sup> transient decrease in

<sup>1</sup> Locke, A., *J. Immunol.*, 1939, **30**, 159-365.

<sup>2</sup> Locke, A., and Main, E. R., *J. Immunol.*, 1939, **30**, 173.

TABLE I  
Transient Decrease in Limitation to Movement Imposed by Muscle Spasm and Pain, in Rheumatoid (Atrophic) Arthritis, Following Inhalation of 10 to 15% CO<sub>2</sub>

Subject	No of consecutive daily inhalations given	Extent of freedom permitted in the movement described	Before CO <sub>2</sub> inhalation		After		Indication of a decrease in pain as appraised by Subject, Observer	Hr duration of the combined effect
DB	1	Active flexion, knee	80°	complete	+	+		10
BB	1	" " knees	0°	0°				
	8			15, 20°				
	14			16, 20°				
	1	Upward motion, from bed	0 in	3, 1, 6, 0 in	+	+		0 12
	8	shoulders, elbows, heels		4, 6, 10, 4 in				> 12
	14			6, 7, 10, 5, 7 in				retained
MO	1	Active flexion, left elbow	5°	10°				
	6			30°				
	34			40°				
	1	" " right knee	10°	10°	+	+		2
	16			20°				6 12
	34			35°				retained
	1	Raising right arm	110°	170°				
	1	Separation, teeth	¼ in	¼ in			(unable to eat solid food)	
	1		40, 50°	50, 60°			(eating solid food)	6 12
EW	1	Passive flexion, knees	0°	8°	+	+		1 2
LD	1	" " left elbow	partial	complete	+	+		8
JP	1	Clenching hand	limps	still limps	+	+		
HG	1	Walking	walking normally		+	+		
DN	8	Motility of arms and shoulders increased			+	+		0
VW	1	Motility of fingers increased			+	+		6 12
RF	1	" " " "			+	+		

TABLE II  
A Related but Less Frequently Elicited, Less Definite and Less Specific Effect in Conditions with Joint Stiffness, Pain or Incapacitation not Caused by Rheumatoid (Atrophic) Arthritis

Extent of freedom permitted in the movement described						Hr duration of the combined effect
No of consecutive daily inhalations given	Before CO <sub>2</sub> inhalation		After		Indication of a decrease in pain as appraised by Subject, Observer	
Osteo arthritis						
CB	1	Sitting up	to 60°	to 90°	+	6 12
NC	1	Passive flexion, knees	120, 125°	130, 137°	+	1
PM	1	No measurable change			+	6 12
AR	1	"			+	
Fibrositis						
LM	1	"			+	1
LC	1	"			+	
OL	1	"			+	
Arthropalms, post operative						
CA	1	Active flexion, elbow	0°	15°	+	7
Joint Infection						
CT	1	Mobility of legs increased			+	3 4
Chronic knee						
VB	1	Ascending stairs	0	0	+	3 4
	7			+		>13

TABLE I  
Transient Decrease in Limitation to Movement Imposed by Muscle Spasm and Pain, in Rheumatoid (Atrophic) Arthritis, Following Inhalation of 10 to 15% CO<sub>2</sub>

Subject	No of consecutive daily inhalations given	Extent of freedom permitted in the movement described		Indication of a decrease in pain as appraised by Subject, Observer	Hr duration of the combined effect
		Before CO <sub>2</sub> inhalation	After		
DB	1	Active flexion, knee	80°	+	10
BB	1	" " knees	0°	+	
	8				
	14		complete 0°		
	1	Upward motion, from bed	15,20°		
	8	shoulders, elbows, heels	15,20°		
	14		3, 1, 0, 0 in	+	6 12
MC	1	Active flexion, left elbow	4, 0, 10, 4 in		> 12
	6		6, 7, 10, 5, 7 in		retained
	34		10°		
	1	" " right knee	30°		
	16		40°		
	34		10°	+	2
	1	Raising right arm	20°		6 12
EW	6	Separation, teeth	35°		retained
LD	1	Passive flexion, knees	110°		
JP	1	" " left elbow	1/4 in	(unable to eat solid food)	
HG	1	Clenching hand	1/4 in	(eating solid food)	
	1	Walking	50, 60°	+	6 12
	8		8°	+	1 2
DN	1	Motility of arms and shoulders increased	complete	+	8
VW	1	Motility of fingers increased	still limps	+	
RF	1	" " " "	walking normally	+	6 12

mistakable increase in exercise tolerance was obtained. Kerr<sup>9</sup> and associates have used CO<sub>2</sub> to combat anxiety complex, effort syndrome and related conditions precipitated by hyperventilation and characterized by impaired ability to relax and to utilize relaxation as a means of conserving and accumulating strength. Over-breathing is a constant hazard when pain<sup>10</sup> is combined with inactivity. Maintenance of an adequate circulation is the major defense not only against low-grade infection<sup>1</sup> but also against atrophy and the type of structural change associated with arthritis.<sup>11</sup>

The observations reported above suggest, but do not establish, the possibility that CO<sub>2</sub>-impoverishment may be a factor in the development and progress of atrophic arthritis. Work is under way on the larger problem of therapy. No conclusive progress in that direction has been obtained or is herewith implied.

*Summary* A transient, small but measurable and significant decrease in limitation to motion was observed in 10 persons with atrophic arthritis following inhalation of 10 to 15% CO<sub>2</sub> (in O<sub>2</sub>) for a length of time (2 to 5 min) sufficient to induce a feeling of warmth and breathlessness. An equivalently definite and regularly elicited effect was not observed in 10 additional persons with limitations due to conditions (osteo-arthritis, fibrositis, etc.) not classifiable as atrophic arthritis. Transient decrease in sensitivity to cold and in susceptibility to histamine was observed in rabbits during inhalation of CO<sub>2</sub> in O<sub>2</sub>.

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<sup>9</sup> Kerr, W. J., Dalton, J. W., and Gleebe, P. A., *Ann Int Med.*, 1937, **11**, 961, Soley, M. H., and Shock, N. W., *Am. J. Med Sci.*, 1938, **106**, 840.

<sup>10</sup> Henderson, Y., *Am J Physiol*, 1909 **10**, **25**, 310.

<sup>11</sup> Goldhaft, A. D., Wright, L. M., and Pemberton, R., *Am J Med Sci.*, 1930, **180**, 386.



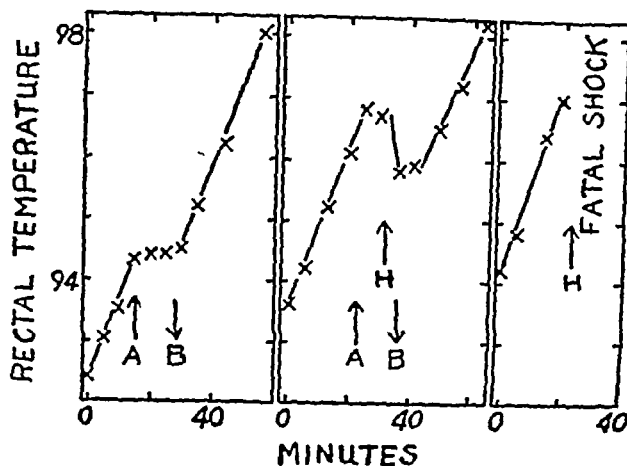


FIG 1.

Effect of inhaled 10%  $\text{CO}_2$  (in  $\text{O}_2$ ) on responsiveness to stimulus and on susceptibility to histamine shock in chilled rabbits. The crosses indicate the course of spontaneous temperature recovery following chilling to the beginning temperatures indicated. A marks the time at which inhalation was begun, B marks the time of discontinuance, 0.8 mg of histamine was given, intravenously, at the time marked H.

susceptibility to histamine. Carbon dioxide raises the threshold of sensitivity to stimulus<sup>3</sup> to an extent capable of producing anesthesia<sup>4</sup> on inhalation for 1 minute or longer in concentrations exceeding 20%. Histamine is the possible chemical mediator of pain.<sup>5</sup>

Mixtures of carbon dioxide with air or oxygen have been given over long periods of time in amounts and concentrations in excess of those given the subjects in Tables I and II, without ill effect and with many indications of beneficial effect (improvement in peripheral circulation).<sup>6,7</sup> Marked relaxation was observed by Loevenhart,<sup>8</sup> during administration of  $\text{CO}_2$  to a group of psychotics for the purpose of rousing them from catatonia. Spontaneous movements of the extremities occurred. The eyes lost their fixed, staring quality and undertook purposeful movement. Carbon dioxide was given by Henderson<sup>9</sup> for the purpose of obtaining increase in collateral circulation in angina pectoris and intermittent claudication. Un-

<sup>3</sup> Hettwer, J. P., *Am J Physiol*, 1938, **122**, 275.

<sup>4</sup> Leake, C. D., and Waters, R. M., *J Pharm. and Exp Therap*, 1928, **33**, 280.

<sup>5</sup> Rosenthal, S. R., and Minard, D., *J Exp Med*, 1939, **70**, 415.

<sup>6</sup> Henderson, Y., *Am Heart J*, 1930 **31**, 6, 548.

<sup>7</sup> Dautrebande, L., et al., *Ann. physiol. psychocohim. biol.*, 1938, **14**, 516, Klingman, T., *Ann. Int. Med.*, 1939, **13**, 677.

<sup>8</sup> Loevenhart, A. S., Lorenz, W. F., and Waters, R. M., *J Am Med Assn*, 1929, **92**, 880.

mistakable increase in exercise tolerance was obtained. Kerr<sup>9</sup> and associates have used CO<sub>2</sub> to combat anxiety complex, effort syndrome and related conditions precipitated by hyperventilation and characterized by impaired ability to relax and to utilize relaxation as a means of conserving and accumulating strength. Over-breathing is a constant hazard when pain<sup>10</sup> is combined with inactivity. Maintenance of an adequate circulation is the major defense not only against low-grade infection<sup>1</sup> but also against atrophy and the type of structural change associated with arthritis.<sup>11</sup>

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<sup>9</sup> Kerr, W. J., Dalton, J. W., and Glibe, P. A., *Ann Int Med*, 1937, **11**, 961, Soley, M. H., and Shock, N. W., *Am J Med Sci.*, 1938, **190**, 840.

<sup>10</sup> Henderson, Y., *Am J Physiol*, 1909 **10**, **25**, 310.

<sup>11</sup> Goldhaft, A. D., Wright, L. M., and Pemberton, R., *Am J Med Sci*, 1930, **180**, 386.

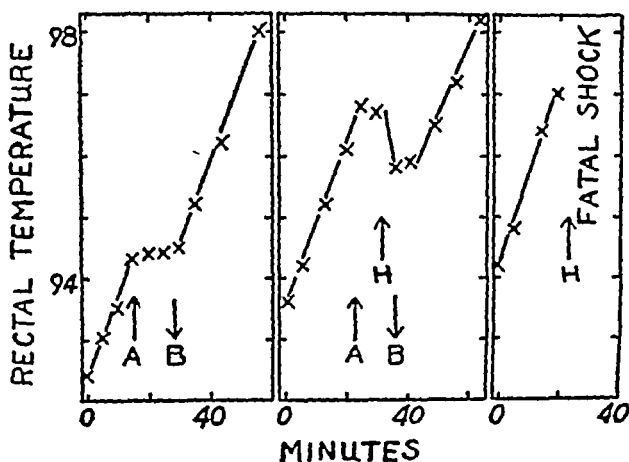


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<sup>4</sup> Lenke, C. D., and Waters, R. M., *J. Pharm. and Exp. Therap.*, 1928, **33**, 280.

<sup>5</sup> Rosenthal, S. R., and Minard, D., *J. Exp. Med.*, 1939, **70**, 415.

<sup>6</sup> Henderson, Y., *Am. Heart J.*, 1930 **31**, **6**, 548.

<sup>7</sup> Dautrebande, L., et al., *Ann. physiol. physicochim. biol.*, 1938, **14**, 516, Klingman, T., *Ann. Int. Med.*, 1939, **18**, 677.

<sup>8</sup> Loevenhart, A. S., Lorenz, W. F., and Waters, R. M., *J. Am. Med. Assn.*, 1929, **92**, 880.

tion The bovine plasma was prepared similarly, (10 cc of a 2.5% sodium citrate solution per 100 cc of blood) the blood, after collection from a living animal by venipuncture, being placed in centrifuge tubes with a capacity of 250 cc Centrifugation was carried out for one hour at 2500 revolutions per minute The supernatant plasma was pipetted off into sterile flasks for storage in the ice box (temperature 5°C) where it was allowed to remain for a week or more before being used The sterility of the bovine plasma was determined by culture on veal infusion broth before administration Several batches of plasma were passed through a Berkefeld filter as an added precaution, but extensive trial showed that this procedure was not necessary to insure sterility Sulfanilamide, 0.2 gram to a liter of plasma, was used in some instances as a preservative (Novak)<sup>3</sup> Through the cooperation of the Department of Veterinary Medicine of the University of Minnesota, it was possible to secure fairly large weekly stores of bovine blood from cows that were free from tuberculosis and Bang's disease

During the period of the study, the patients were on a protein-free diet Tea, orange juice, nectar and water were allowed by mouth to supplement intravenous administration of 5 or 10% glucose solution Enough carbohydrate was given in this manner to afford a caloric intake of about 1200 calories daily and to avoid ketosis

The total daily output of urine passed was preserved with toluene Daily determinations of the total urinary nitrogen<sup>4</sup> non-protein nitrogen urea<sup>5</sup> nitrogen and sugar were made At the beginning of the study and at the end, and at intervals of 2 to 3 days between, values for plasma proteins non-protein nitrogen<sup>5</sup> and blood urea nitrogen<sup>6</sup> and chlorides<sup>7</sup> were determined The specific gravity of the blood of the recipients was kindly determined for us by Dr C J Bellis by the falling drop method<sup>8</sup> The weight of the patient was determined at intervals through the period of study

As indicated in Table I, the usual procedure was to allow the excretion of nitrogen in the urine to reach a basal level, the patient being supported by a carbohydrate intake only in the 3- to 5-day

<sup>3</sup> Novak, M, *J A M A*, 1939, 113, 2227

<sup>†</sup> It is a real pleasure to acknowledge here our gratitude for helpful assistance to the late Dr C P Fitch and to Dr Willard L Boyd Professors of Veterinary Medicine

<sup>4</sup> Koch, F C and McMeekin, T L, *J Amer Chem Soc*, 1924, 46, 2066

<sup>5</sup> Folin, O, and Wu, H *J Biol Chem*, 1919, 38, 81-111

<sup>6</sup> Karr, W G, *J Lab and Clin Med*, 1924, 9, 329

<sup>7</sup> Cuvett, T W, and Holdridge C F *J Lab and Clin Med* 1933, 18, 944

<sup>8</sup> Barbour, H F and Hamilton, W F *J A M A* 1927 88, 91

## Intravenous Administration of Bovine and Human Plasma to Man Proof of Utilization \*

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Minneapolis, Minn*

In this and other clinics, human plasma has been given intravenously in preparing patients with obstruction at the gastric outlet, exhibiting the effects of hypoproteinemia, for operation Whipple<sup>1</sup> and his associates have demonstrated that dog plasma administered intravenously to dogs is utilized In this study it is proposed to indicate that (1) Human plasma administered intravenously to patients is retained and utilized, (2) plasma presents distinct advantages over whole blood for the purpose of maintaining nitrogen equilibrium and to elevate depleted plasma proteins in starvation states, (3) bovine plasma may be given intravenously to man in fairly large quantities, it too is retained and utilized

We wish to make it clear that the intravenous administration of bovine plasma to man has not been established as a safe routine hospital procedure The data submitted herewith afford proof, however, that bovine plasma may be administered intravenously to some patients in fairly large quantities When the possibilities and limitations of the method become understood well enough, it is not unlikely that the method may become a practical hospital procedure useful in civil as well as in war surgery for various purposes having to do with contracted blood volumes and protein stores

*Method* All the patients upon whom metabolic tests were made in this study and to whom human and later bovine plasma was given, were afflicted with cancer and presented some of the effects of protein starvation All patients, however, were essentially afebrile The human plasma was obtained from the surgical blood bank of citrated blood in the hospital, the cells being removed by centrifuga-

\* The researches presented herewith were supported by grants of the Graduate School of the University of Minnesota and by a grant for technical assistance by the Work Projects Administration, Official Project No 665 71 3 69, Sub project No 258

<sup>1</sup> Pommerenke, W T, Slavin, H B, Karner, D H, and Whipple, J G, *J Exp Med*, 1935, **61**, 283

<sup>2</sup> Daft, F S, Robschtein-Robbins, F S, and Whipple, J G, *J Biol Chem*, 1938, **123**, 87

interval The effect of the administration of human blood or plasma upon the excretion of nitrogen was noted first, then, similar studies were made during the administration of bovine plasma The daily administered amounts of human blood or plasma and bovine plasma indicated in the table were given in 2 divided doses (morning and evening) An intracutaneous injection of 0.05 cc of bovine plasma was first made as a test If no enlargement of the wheal was observed at 20 and 40 minutes, the test was interpreted as being negative and 2 cc of bovine plasma was injected intravenously, before larger amounts were given None of the patients were diabetic and the urinary excretion of sugar represents spillage from too rapid administration of 10% glucose solution

*Results* Representative results are indicated in the table For purposes of conserving space, the detailed information in Cases 2, 4, 5 and 6 are omitted in the table Only Cases 1, 5 and 6 failed to receive bovine plasma intravenously Case 2 received 750 cc of bovine plasma over a 4-day interval, Case 4 received 1000 cc of bovine plasma and the daily urinary excretion of nitrogen was definitely lower than in the 4 days preceding during which 2000 cc of whole human blood was given Tests for protein in the urine were uniformly negative, in all instances, the total urinary nitrogen was accounted for by non-protein nitrogen The urinary nitrogen studies in Case 1 (see table) suggest a definitely greater urinary excretion of nitrogen during intravenous administration of whole human blood than when human plasma was given This observation appears to be borne out in other instances, *viz.*, that administration of whole human blood decreases the urinary excretion of nitrogen In Case 5, after 4 days of protein starvation, the urinary excretion of nitrogen fell to 1.26 g per day, 500 cc of whole human blood was then given intravenously for 6 days On the 5th day the urinary excretion of nitrogen was 9.12 g, on the 6th day 8.3 g In Case 1, over the 5-day period that 2250 cc plasma was given (protein content 7 g per 100 cc), a total of 157.5 g of protein or 25.3 g of nitrogen was introduced During this same time 19.34 g of nitrogen was excreted in the urine, indicating a nitrogen retention of 6 g

In Case 3 (see table), the average daily urinary output of nitrogen was 7.61 g after 3 days of daily administration of 500 cc of whole human blood intravenously, preceded in turn by 3 days of no exogenous intake of protein Accompanying 4 days of administration of bovine plasma during which time 800 cc had been given, the daily urinary nitrogen excretion was 4.34 g, suggesting that the intravenously administered protein was being stored somewhere in the



*Reactions* Bovine plasma has been given intravenously in small amounts (2 to 10 cc) to a larger number of patients who will be reported upon in a later study. It may be said here that of 66 patients so injected reactions were noted in 10 cases (15%). Nine of these reactions were classified as mild to moderate in severity. In a patient with an inoperable carcinoma of the rectum, having a history of asthma dating back 20 years, a severe anaphylactoid type of reaction with severe dyspnoea attended the intravenous injection of 2 cc of bovine plasma. The patient responded satisfactorily to intravenous injections of epinephrin and aminophyllin. The patient had no late effects.

A thermal reaction was noted in Case 3. In 3 of the patients given large quantities of bovine plasma upon whom metabolic studies were made, a generalized urticaria developed 5 to 6 days after the administration of bovine plasma was begun. The urticaria lasted usually 3 to 4 days and was fairly satisfactorily relieved by epinephrine. There was no joint manifestations.

Three patients in the series were given a single intravenous injection of bovine plasma in the amounts of 100, 50 and 25 cc after 8, 7 and 48 days respectively had elapsed since the last injection. No reactions attended these injections.

*Discussion* It would appear that the use of bovine plasma for the treatment of clinical states in which contracted blood volumes or decreased protein stores are present may have real promise. Before intravenous use of bovine plasma can be recommended for clinical usage, however, it is important to determine with some precision what the limitations of the method are with reference to safety of administration. It may prove that partition of the proteins in the plasma, *viz.*, administration of the albumen or the globulin fraction alone may prove more useful than the whole plasma.

*Conclusions* 1 Human plasma administered intravenously is retained and utilized. A patient to whom a daily average of 450 cc of plasma was given over a 5-day period was maintained in positive nitrogen balance. 2 Human plasma appears to be a better agent than blood to employ to maintain nitrogen equilibrium in starvation states. 3 Bovine plasma can be given intravenously to man in fairly large quantities; it is retained and apparently utilized.



body Determination of the protein content of bovine plasma was made on 4 batches Values between 7 and 8 g per 100 cc were uniformly found Case 7 (see table) received a total of 1500 cc of bovine plasma over a 5-day period, without increasing the daily urinary excretion of nitrogen appreciably over what it had been after daily administrations of 500 cc of whole human blood over a 4-day period

In no instance did we succeed in keeping the patient in nitrogen balance attending intravenous injection of bovine plasma In Case 3, there was an excretion of 7.1 g more of nitrogen in the urine over the intake, during the 4-day period of the study Similarly in Case 7, there was a negative nitrogen balance of 9.968 g for the 5-day period However, in Case 3, the average daily excretion of nitrogen was definitely less than the basal level for the control period when there was no exogenous intake of nitrogen While in Case 7, the daily average excretion of nitrogen in the urine, during the period when bovine plasma was given, was less than the basal level of the preliminary 4-day period of protein starvation Further, few significant increases in the non-protein nitrogen of the blood occurred during the administration of bovine plasma The relationship between the total non-protein nitrogen and the urea of both blood and urine showed no significant alteration suggestive of abnormal catabolism of protein

Inasmuch as Case 3 was given only an average of 2.5 g of nitrogen per day and Case 7, 3.84 g, it is reasonable to infer that not enough protein (bovine plasma) was given to maintain a positive nitrogen balance In Case 1, a positive nitrogen balance was maintained with human plasma, an average of 5.06 g of nitrogen being given each day of the 5-day period of study Inasmuch as enough bovine plasma was not given to maintain nitrogen equilibrium, one could not reasonably expect that the plasma proteins of these patients would be increased

To a patient with a bleeding gastric ulcer upon whom no metabolic studies were made, 25 and 100 cc of bovine plasma were injected intravenously on 2 successive days without reaction Two days later when bleeding to a shock level occurred (blood pressure 80/40), 300 cc of bovine plasma was given over an hour's time, during the next 2.5 hours an additional 400 cc was given—all without reaction, the blood pressure having risen after the initial injection of 300 cc to 130/90, at which level it was maintained This was the largest amount of bovine plasma injected over a relatively short period of time

TABLE I.

Group	12 hr	24 hr	% Mortality at 48 hr	72 hr	96 hr
A	20	100	—	—	—
B	0	0	10	20	20
C	20	100	—	—	—
D	10	100	—	—	—

Group D 10 mice Experimental series One cc of a 1% acacia suspension of N<sup>1</sup>-dodecanoylsulfanilamide administered orally 1 hour and 24 hours after infection

The evidence elicited from the study of the absorption and distribution of the drug suggested that it would be necessary to administer the drug together with fats capable of being absorbed from the gastrointestinal tract if any therapeutic effect was to be demonstrated. Accordingly all subsequent experiments involved the administration of the drug orally in olive oil or milk and oil suspensions. When administered in this manner, the drug appeared to be somewhat more effective against beta hemolytic streptococcal infections than sulfanilamide itself.

*Experiment 2* All animals infected by the intraperitoneal administration of  $1 \times 10^{-6}$  cc of a 16-hour culture of C-203

Group A 100 mice Untreated controls

Group B 250 mice Sulfanilamide controls One cc of a 1% acacia suspension administered orally 1 hour and 24 hours after infection

Group C 200 mice Experimental series One cc of a 1% olive oil solution of N<sup>1</sup>-dodecanoylsulfanilamide administered orally 1 hour and 24 hours after infection

Group D 100 mice Experimental series One cc of a 1% suspension of N<sup>1</sup>-dodecanoylsulfanilamide in milk and oil administered orally 1 hour and 24 hours after infection

As is the case with sulfanilamide itself, N<sup>1</sup>-dodecanoylsulfanilamide shows no protective action against overwhelming infections.

*Discussion* An exhaustive pharmacological study of this drug is being carried out at the present time and the results will be reported in

TABLE II.

Group	12 hr	24 hr	% Mortality at 48 hr	72 hr	96 hr
A	26	100	—	—	—
B	0	2	8	10	16
C	0	0	2	4	4
D	0	1	1	1	1

# N<sup>1</sup>-Dodecanoylsulfanilamide I Experimental Infections with Beta Hemolytic Streptococci

DAVID R. CLIMENKO AND R. L. SCHMIDT (Introduced by Harry Gold)

*From the Pharmacological Laboratory, The Calco Chemical Company, Cold Spring Harbor*

The chemical properties of N<sup>1</sup>-dodecanoylsulfanilamide have been described by Crossley, Northey and Hultquist<sup>1</sup> and the absorption, distribution, and toxicity of the drug will be discussed in another paper of this series. The present report deals with the chemotherapeutic effects of the drug in experimental infections of mice with beta hemolytic streptococci.

While 3 strains of beta hemolytic streptococci, C-203,\* SH-1685,† and "Todd",‡ have been employed in this work, the results have been so consistent that the report will only deal with the effects on C-203. Virulence titrations of a 16-hour culture of this organism grown on fresh beef infusion, whole blood media give 100% mortality figures within 24 hours for concentrations down to  $1 \times 10^{-6}$  cc and 100% mortality figures within 72 hours for concentrations down to  $1 \times 10^{-7}$  cc. These virulence figures refer to young mice of the Swiss strain weighing between 10-12 g. The absorption of N<sup>1</sup>-dodecanoylsulfanilamide from the gastrointestinal tract seems to be dependent on the absorbable lipoids present in the gut. This fact manifested itself to us in our earlier experimental work when it appeared that the drug had no protective action in streptococcal infections after it had been administered either orally or subcutaneously in acacia suspension. The following experiment illustrates this point.

*Experiment 1* All animals infected by the intraperitoneal administration of  $1 \times 10^{-6}$  cc of a 16-hour culture of C-203

Group A 10 mice Untreated controls

Group B 10 mice Sulfanilamide controls 1 cc of a 1% acacia suspension of sulfanilamide administered orally 1 hour and 24 hours after infection

Group C 10 mice Experimental series One cc of a 1% acacia suspension of N<sup>1</sup>-dodecanoylsulfanilamide administered subcutaneously 1 hour and 24 hours after infection

<sup>1</sup> Crossley, M. L., Northey, E. H., and Hultquist, M. E., *J. A. C. S.*, 1939, **61**, 2950

\* From the Johns Hopkins Hospital, Baltimore, Md.

† From the National Institute of Health, Washington, D. C.

‡ From the Lilly Laboratories, Indianapolis, Ind.

these observations, but Kolmer, Raiziss and Rule<sup>6</sup> and Smithburn<sup>7</sup> have been unable to reproduce these results. Smithburn produced an experimental tuberculous meningitis, and administered the drug intraperitoneally, while Kolmer and his associates administered the drug intramuscularly. We have always found that oral administration of sulfanilamide is the most effective method of administration.

The fatty acids of chaulmoogra oil have been suggested for the treatment of tuberculous infections for some time. Walker and Sweeney<sup>8</sup> made this suggestion in 1920, while Rogers<sup>9</sup> and Burgess<sup>10</sup> reported clinical successes in the treatment of the dermal manifestations of tuberculous infections with hydnocarpic acid esters. Walker and Sweeney pointed out that chaulmoogric acid was not capable of penetrating the wall of the tubercle, and Kolmer, Davis and Jager<sup>11</sup> demonstrated that chaulmoogric acid had no inhibiting effect on the development of experimental tuberculous infections in guinea pigs.

The combination of a long chain fatty acid with sulfanilamide, combining the fat solubility of the one with the penetrating properties of the other, might provide a more effective agent in controlling experimental tubercular infections in guinea pigs than was available in sulfanilamide itself or in any member of the fatty acid series itself. It was with this specific purpose in mind that N<sup>1</sup>-dodecanoylsulfanilamide was synthesized.

It was first demonstrated that N<sup>1</sup>-dodecanoylsulfanilamide was capable of inhibiting the growth of human tubercle bacilli *in vitro*. A series of flasks containing beef infusion, glycerine, dextrose broth were set up to contain N<sup>1</sup>-dodecanoylsulfanilamide in concentrations of 10 mg/100 cc, 20 mg/100 cc, and 100 mg/100 cc. A similar preparation was made containing sulfanilamide at a concentration of 100 mg/100 cc. A series of untreated control flasks were also set up. All flasks were inoculated with the H 37 strain of human tubercle bacilli and incubated at a temperature of  $37.5^{\circ}\text{C} \pm 2^{\circ}\text{C}$  for a period of 90 days. Concentrations of 10 mg/100 cc of N<sup>1</sup>-dodecanoylsulfanilamide in the culture medium inhibited growth over this period, while a similar inhibition was produced by a concentration of 100 mg/100 cc of sulfanilamide. The controls grew out luxuriously.

<sup>6</sup> Kolmer, J. A., Raiziss, G. W., and Rule, A. M., *PROC SOC EXP BIOL AND MED*, 1938, **30**, 581.

<sup>7</sup> Smithburn, K. C., *PROC SOC EXP BIOL AND MED*, 1938, **38**, 574.

<sup>8</sup> Walker, E. L. and Sweeney, M. A., *J Infect Dis*, 1920, **26**, 238.

<sup>9</sup> Rogers, L., *Brit Med J.*, 1933, **1**, 47.

<sup>10</sup> Burgess, W., *Brit Med J*, 1935, **2**, 835.

<sup>11</sup> Kolmer, J. A., Davis, L. C. and Jager, R., *J Infect Dis*, 1921, **28**, 265.

the immediate future. It has been suggested<sup>2</sup> that the activity of the drug is dependent on its breakdown with a resultant liberation of sulfanilamide. Our present direct evidence shows that no such breakdown occurs in the gastrointestinal tract. Indirect evidence from studies on therapeutic efficacy<sup>3</sup> indicates that this N<sup>1</sup>-acyl compound is more active than sulfanilamide on a molecular basis.

*Results* 1 N<sup>1</sup>-dodecanoylsulfanilamide, when administered in oil to mice, shows marked therapeutic efficacy against beta hemolytic streptococci. 2 This therapeutic effect is lost when the drug is administered in an aqueous medium.

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## N<sup>1</sup>-Dodecanoylsulfanilamide II Experimental Infections with *Mycobacterium tuberculosis*

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It was pointed out<sup>1</sup> that N<sup>1</sup>-dodecanoylsulfanilamide, when administered in fatty menstua, exerted a more potent therapeutic effect against experimental beta-hemolytic streptococcal infections than sulfanilamide. This compound, whose chemical and physical properties have been described,<sup>2</sup> differs from sulfanilamide only in so far as a long chain fatty acid has been substituted for an H atom in the N<sup>1</sup> position. This gives to the compound a very high degree of fat solubility.

A new impetus has been lent to the subject of the chemotherapy of tuberculous infections by the report of Rich and Follis<sup>3</sup> that sulfanilamide is capable of inhibiting the development of the tuberculous process in guinea pigs after the experimental infection with human tubercle bacilli. Greey and his associates<sup>4, 5</sup> have repeated

<sup>1</sup> Marshall, Jr., E. K. Personal communication.

<sup>2</sup> Feinstein, W. H., Wolff, R. and Williams, R. D. To be published.

<sup>3</sup> Rich, A. R. and Follis, A. H., *Bull. Johns Hopkins Hosp.*, 1938, **62**, 77.

<sup>4</sup> Greey, P. H., Campbell, H. H., and Colly, A. W., *Proc. Soc. Exp. Biol. and Med.*, 1938, **39**, 22.

<sup>5</sup> Greey, P. H., Boddington, G. D. H., and Little, M. H., *Proc. Soc. Exp. Biol. and Med.*, 1939, **40**, 448.

Animals of Group D showed localized tubercular lesions at the site of infection and involvement of the adjacent lymphatics. Practically all of these animals developed discharging purulent infections at the injection site. They showed no gross splenomegaly, liver involvement or kidney involvement. There was no macroscopic evidence of generalized tuberculous lymphadenitis; in no instance did the disease process extend beyond the periaortic glands at the bifurcation of the aorta.

**Conclusions** 1 N<sup>1</sup>-dodecanoylsulfanilamide inhibits the growth of tubercle bacilli *in vitro* at a concentration of 10 mg/100 cc in beef infusion-dextrose-glycerine media over a period of 90 days. 2 N<sup>1</sup>-dodecanoylsulfanilamide inhibits the development of the tuberculous process in guinea pigs infected subcutaneously with a human strain of tubercle bacilli.

## 11284 P

### Production of Pneumonia in Rats by Intravenous Injection of Pneumococci \*

ALICE H. KEMPF AND W. J. NUNGESTER

*From the Hygienic Laboratory, University of Michigan, Ann Arbor, Mich*

Rake<sup>1</sup> demonstrated that pneumonia could be produced in mice when the pneumococci were introduced by the intravenous route. The important factors were the strain and dose of the organism and the breed of mice. Twelve of 87 mice had macroscopic lesions, 6 of the 87 failed to show any microscopic lesions. The pathology of the various stages encountered was clearly described.

Prior to this work, with a few exceptions, intravenous injection of pneumococci has failed to give rise to pneumonia. Although it has long been postulated that the origin of the infection might be by way of the blood stream, there has been little evidence to support it.

Employing the Schwartzman phenomenon, Witebsky, Neter, and Ward<sup>2</sup> were able to localize pneumococci injected intravenously in dermal lesions of rabbits.

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\* These studies were aided by a grant from the Horace H. Rackham School of Graduate Studies.

<sup>1</sup> Rake, Geoffrey. *J. Exp. Med.* 1936, **63**, 191.

<sup>2</sup> Witebsky, Ernest and Neter, Erwin. *Proc. Soc. Biol. and Med.* 1938, **38**, 187.

The *in vivo* work was carried out on guinea pigs. A group of 80 animals, weighing between 350-400 g were infected by the subcutaneous administration of 1 mg of the H 37 strain of human tubercle bacilli, administered in the region of the groin. These animals were then divided into 4 groups of 20 as follows: Group A Untreated controls; Group B Sulfanilamide series 100 mg sulfanilamide in 1% acacia suspension administered daily by stomach tube for 45 days. Treatment initiated immediately after infection; Group C Experimental series 100 mg N<sup>1</sup>-dodecanoylsulfanilamide in 2% olive oil solution administered daily by stomach tube for 45 days. Treatment initiated immediately after infection; Group D Experimental series 100 mg N<sup>1</sup>-dodecanoylsulfanilamide in 2% olive oil solution administered daily by stomach tube for 40 days. Treatment initiated 5 days after infection.

All animals in Group A developed the classical signs of tuberculous infection with human strain organisms. Tubercles formed at the site of inoculation, and in most instances, discharged through a secondarily infected sinus. These animals developed generalized tuberculous peritonitis and lymphadenitis with gross splenomegaly and liver involvement. Pulmonary involvement was rarely seen. With the exception of those animals sacrificed 30 days after infection for postmortem examination, all members of this group died within 65 days.

Animals of Group B developed localized tubercular lesions at the site of injection, which rarely showed secondary infections. Animals sacrificed at the end of 30 days for comparison with untreated controls show very slight generalized disease. The splenomegaly in this series was not nearly as pronounced as in the case of the controls and the glandular involvement was considerably less extensive. The results were essentially similar to those described by Rich and Follis and by Greey and his associates.

Animals of Group C showed localized tubercular lesions at the site of infection, which in some instances spread by direct contact to involve a considerable area of tissue. One animal (No. 51 in the series) developed a tuberculous orchitis, but showed no signs of generalized tuberculous lymphadenitis or peritonitis. A large proportion of these animals developed secondary infections at the site of injection, sinuses formed, and heavy purulent material was discharged. In most instances the secondary organisms were staphylococci. Animals of this group sacrificed 120 days after infection showed no gross splenomegaly as compared with the untreated controls. In no instance was there macroscopic evidence of tuberculous peritonitis or tubercles in the liver or kidneys.

Animals of Group D showed localized tubercular lesions at the site of infection and involvement of the adjacent lymphatics. Practically all of these animals developed discharging purulent infections at the injection site. They showed no gross splenomegaly, liver involvement or kidney involvement. There was no macroscopic evidence of generalized tuberculous lymphadenitis; in no instance did the disease process extend beyond the periaortic glands at the bifurcation of the aorta.

**Conclusions** 1 N<sup>1</sup>-dodecanoylsulfanilamide inhibits the growth of tubercle bacilli *in vitro* at a concentration of 10 mg/100 cc in beef infusion-dextrose-glycerine media over a period of 90 days. 2 N<sup>1</sup>-dodecanoylsulfanilamide inhibits the development of the tuberculous process in guinea pigs infected subcutaneously with a human strain of tubercle bacilli.

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TABLE I  
Type I Pneumonia Resulting from Intravenous Injection of Pneumococci

Sterile Mucin Intrabronchially* cc	Pneumococci Intravenously cc	No of rats Inoculated	Dead	% of Animals with Macro- scopic Consolidation†
0.1	10 <sup>-1</sup>	4	4	100
0.1	10 <sup>-2</sup>	21	14	62
0.1	10 <sup>-4</sup>	5	2	0
0.1	10 <sup>-5</sup>	3	1	33
0.1	10 <sup>-7</sup>	4	1	25
0.0	10 <sup>-2</sup>	5	4	0
0.1	0.0	5	0	0

\* Animals which did not develop pneumonia were shown to have small atelectatic lesions, evidence of intrabronchial inoculation.

† From one-fourth to an entire lobe was involved.

In our experiments, rats were inoculated intrabronchially with 0.1 cc of sterile mucin according to a technic previously described,<sup>3</sup> immediately thereafter, the animals were injected intravenously (using the tail vein) with various amounts of culture. The data are presented in Table I.

In interpreting these results it must be remembered that these animals received only one inoculation of organisms, many of which were distributed throughout the body and removed by the fixed phagocytic cells. It is noteworthy that in at least one rat, the disease was produced with as small an inoculum as 10<sup>-7</sup> cc of culture.

Although in the past it has been generally impossible to produce pneumonia in experimental animals by intravenous injection of pneumococci, this may be accomplished in rats following intrabronchial inoculation of sterile mucin. The presence of mucus material in the lung or some comparable condition is a necessary predisposing factor for the localization of the pneumococci.

<sup>3</sup> Nungester, W. J. and Jourdonais, L. F., *J. Infect. Dis.*, 1936, **59**, 258.

# Effect of Gelatin on Power of Women to Perform Maximal Anaerobic Work \*

F A HELLEBRANDT, ROZELL RORK AND ELIZABETH BROGDON  
(Introduced by W J Meek )

*From the Department of Physiology University of Wisconsin Medical School*

Glycine is present in gelatin to the extent of 25% Its creatinogenic action is the basis for its use in the treatment of myopathies and simple fatigability (Boothby,<sup>1</sup> Wilder,<sup>2</sup> Tripoli and Beard<sup>3</sup>) However, evidence is accumulating which suggests that although the anaerobic reactions of muscle biochemistry involve creatine, such reactions may be little concerned with the performance of exercise in the steady state (Sacks,<sup>4</sup> Flock, Ingle and Bollman,<sup>5</sup> Millikan<sup>6</sup>) It would, therefore, seem highly desirable and possibly even necessary to use maximal anaerobic work in quantitative studies designed to test the influence of gelatin on muscular fatigue which are based upon the creatine-fixing function of glycine Ray, Johnson and Taylor<sup>7</sup> reported that the daily administration of 60 g of gelatin to each of 6 men for a period of weeks was associated invariably with an increase in work capacity No appreciable effect was demonstrable when comparable doses of gelatin were given to 4 women included in the study The rate of work which produced exhaustion within a few minutes the magnitude of the difference in muscle power exhibited by the male and female subjects and the sex variation in the response to gelatin were all sufficiently unusual to warrant repetition of the observations made on women

*Methods* The subjects of the investigation were 6 young adult women all of whom had had professional training in physical education and were accustomed to severe physical activity The exercise was performed on an electrodynamic brake bicycle ergometer equipped with a graphic voltmeter to record rate of working and the speed of

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\* Supported in part by funds from the Wisconsin Alumni Research Foundation.

<sup>1</sup> Boothby, W M *Proc Staff Meet Mayo Clinic* 1934 **9** 593 600

<sup>2</sup> Wilder, R M. *Proc Staff Meet Mayo Clinic* 1934 **9** 606

<sup>3</sup> Tripoli C J and Beard, H H *Southern Med J* 1935 **31** 662

<sup>4</sup> Sacks, J, *Am J Physiol*, 1935 **122** 215

<sup>5</sup> Flock, E V Ingle D J, and Bollman J L *J Biol Chem* 1939 **129**, 99

<sup>6</sup> Millikan G A *Physiol Rev* 1939, **19** 503

<sup>7</sup> Ray G B Johnson I R and Taylor M M *Proc Soc Exp Biol and Med* 1939 **40**, 157

pedalling (Kelso and Hellebrandt<sup>8</sup>) Load and speed were standardized for each subject Duration of exercise was the independent variable The subject rode to exhaustion, the end-point for exhaustion being inability to maintain the voltage which served as an indicator of the rate of working Fig 1 is a reproduction of a typical record, showing the rapidity with which the voltage rises as the speed of pedalling increases and the constancy with which it is

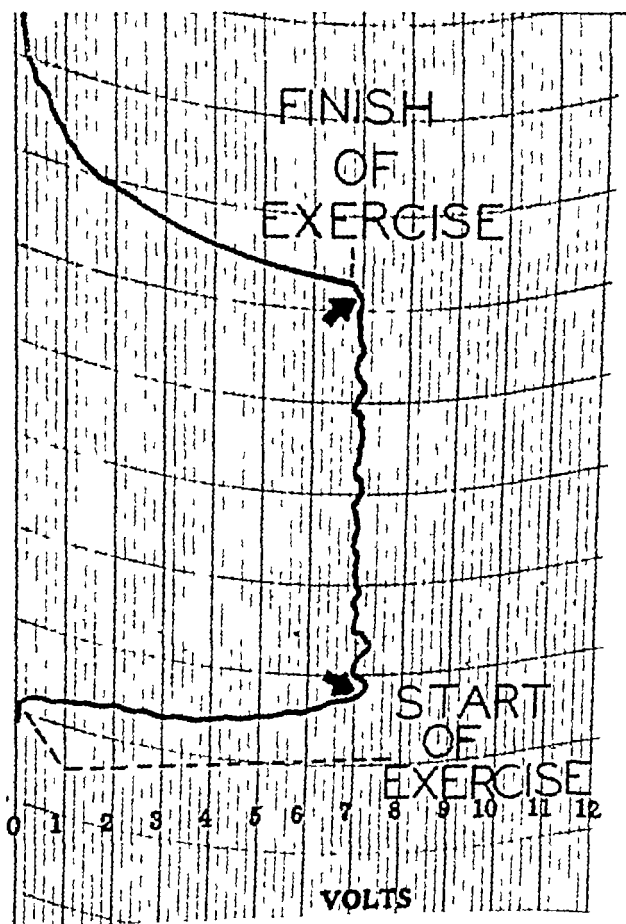


FIG 1

Typical graphic voltmeter record obtained on the electro dynamic brake bicycle ergometer Abscissae mark time in 10 seconds Arrows indicate duration of exercise at the standardized rate of working

<sup>8</sup> Kelso, L. E. A., and Hellebrandt, F. A., *J. Lab. and Clin. Med.*, 1934, 19

maintained once the rhythm is set. Activity ceased abruptly when the rpm necessary to sustain the voltage could no longer be tolerated. Five women worked at a 290 watt *subject output* and one at 220. Exhaustion was usually reached in less than 60 seconds. The duration of the effort in the record illustrated, beginning time at the instant the standard rate of working was achieved, was 45.4 seconds. These findings are in sharp contrast to the data reported by Ray *et al*, whose men and women subjects were rapidly fatigued by an *ergometer output* of 60 and 45 watts respectively.

After a period of preliminary training in the performance of rapid violent work standardized as to severity, gelatin was added to the regular diet and then withdrawn as exercise continued. Five subjects ingested 15 g of glycine *per diem*, administered as 60 g of sheet gelatin dissolved and then allowed to solidify in 250 cc of water to which the juice of one lemon and 2 tablespoons of sugar were added. One subject took half the dose in the form of granular gelatin dissolved in cold fruit juice.

*Results and Their Interpretation* Fig 2 illustrates the results obtained upon R. R., an especially well trained subject who had spent the summer preceding the experiment on a cycling tour. Her

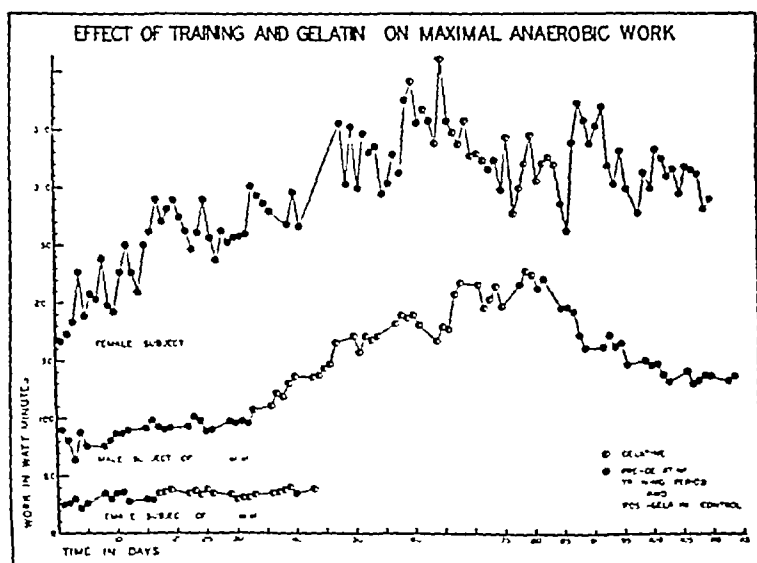


FIG 2

Subject R. R. exercised daily. Voltmeter records were not obtained on 4 days. In reproduction of the curves of Ray *et al*, it was necessary to assume an arbitrary 5 day week so that the number of observations published by them might correspond to the numerical value given for the duration of their experiment in days. This spreads their curve and minimizes the rate of change in power.

ability to perform anaerobic work of extreme severity increased over a period of 65 days. The daily ingestion of gelatin failed to sustain her improving capacity for work. The tendency toward a decrease in power, clearly evident in Fig 2 (R R), may be attributed to "staleness." Though short-lived, the work was so rigorous that recovery may have been incomplete in 24 hours. No athlete can repeat a record-breaking performance daily. Transient increases in power occurred both at the beginning of the administration of gelatin and at the onset of the post-gelatin control period. No matter how well trained the subject, it is never possible to completely eliminate psychic influences in experimentation involving human beings performing work to exhaustion on devices not driven by forces beyond voluntary control.

For purposes of comparison, the only data reported for a woman by Ray, Johnson and Taylor<sup>9</sup> and those obtained on their male subject demonstrating the best effect attributed to gelatin are also reproduced in Fig 2. Henderson and Haggard<sup>9</sup> estimated the maximal power of Yale Olympic oarsmen as 0.56 horse power in a short race lasting 6 minutes. The power developed by R R was 69% of that demonstrated by these men. This compares favorably with Dawson,<sup>10</sup> who estimates that the maximum energy which can be exerted by a woman is approximately 72% that of a man. The power developed by the male subjects of Ray *et al*, was 0.08 horse power a rate of working which could be rapidly exhausting only if carried on at excessively high speeds. Subject R R rode at 98 rpm. In our experience, pedalling rates beyond 120 are self-limiting because of incoordination.

Fig 3 illustrates the effect of introducing gelatin at different phases of the training period. Subject B McL, slight in build and weighing 52 kilos, exhibited the greatest improvement with daily exercise and attained the highest work output in our series. The administration of gelatin relatively early in the experiment may be interpreted as having had no effect upon the trend of the training curve which rose steeply from the beginning to the end of the experiment. Her power to perform work oscillated more widely than that of R E B, whose training curve was closely approximated by our remaining subjects although gelatin was administered to them as early as to B McL. The data presented in Fig 3 suggest that gelatin neither affects the capacity to do more work as a result of repeated

<sup>9</sup> Henderson, Y, and Haggard, H W, *Am J Physiol* 1925 72, 264

<sup>10</sup> Dawson, P M, *The Physiology of Physical Education*, 1935, Baltimore. The Williams & Wilkins Co

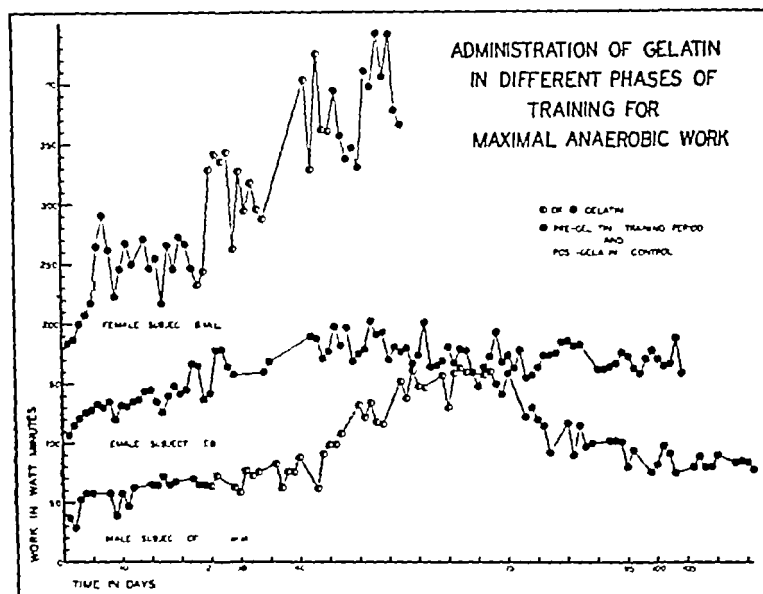


FIG 3

Voltmeter records were not obtained on days in which gaps occur in the curves of B McL and R.E.B. They exercised daily. In this figure as in Fig 2, the 5 day week is arbitrarily used for graphing the data of Ray *et al* against time

activity, nor prevents the development of chronic fatigue (R E B) when the activity is too severe

The gelatin ingested was added to the regular diet increasing its protein content by one gram or more per kilo *per diem*. Wishart<sup>11</sup> showed that the performance of a long distance racing cyclist was best on high protein diets. Mirski and his associates<sup>12</sup> have recently shown that the blood sugar and fuel reserves of the liver laid down on a protein-rich diet behave differently from those deposited on a diet of carbohydrate. Such observations suggest that gelatin might be advantageous in the performance of protracted hard work.

**Summary and Conclusions** Physical exercise maximal anaerobic in type and constant as to the rate of working and speed was performed to the point of exhaustion by 6 healthy young adult women. After a period of preliminary training varying in length in the different subjects, gelatin was added to the diet and its influence upon the time of onset of muscular fatigue was noted. The evidence presented substantiates the following conclusions. Gelatin has no effect

<sup>11</sup> Wishart G M, *J Physiol*, 1934, **82**, 189

<sup>12</sup> Mirski A, Rosenbaum I, Stein L. and Wertheimer, F, *J Physiol* 1938, **92** 48

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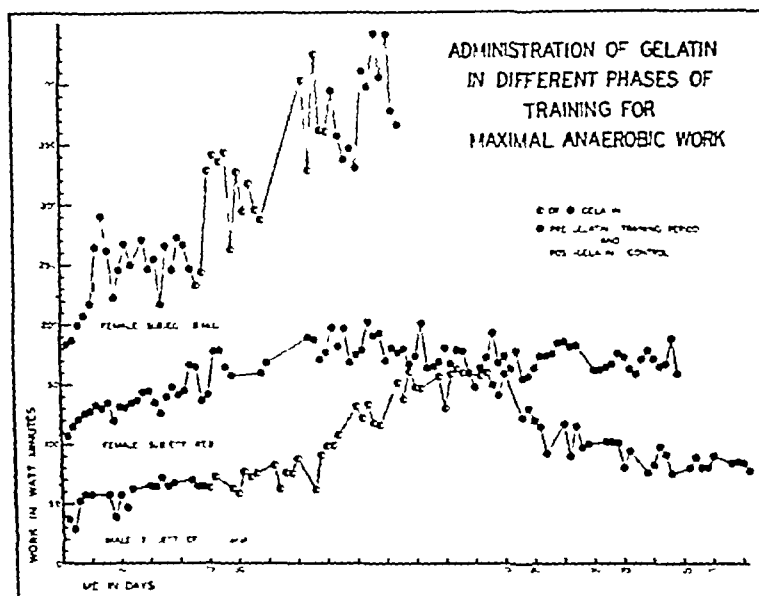


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<sup>11</sup> Wishart, G. M., *J. Physiol.* 1934, 82, 159.

<sup>12</sup> Mirski, A., Rosenbaum, I., Stein, I., and Wertheimer, F., *J. Physiol.* 1958, 102, 48.



upon the capacity of women to perform maximal anaerobic work, nor any apparent effect upon the improvement in power due to training. Gelatin does not prevent the development of "staleness" when brief work of extreme severity is repeated daily over a long period of time. A comparison of the published data of Ray, *et al*,<sup>7</sup> with that of our series of experiments suggests that the increase in work output attributed by these investigators to gelatin may also have been a training effect.

*Acknowledgments* Our best thanks are due to Betty McLaughlin, Mary Stowell, Helene Sanger and Beatrice Hellebrandt for the faithfulness with which they "played themselves out" each time they rode the bicycle ergometer and to Prof. Kelso of the School of Engineering for his analyses of the discrepancies in work evident in the comparison of our observations with those of Ray, Johnson and Taylor.

## 11286

### Further Approach Toward Control of Spontaneous Cancer of Mammary Gland in Mice by Heptyl Aldehyde-Sodium Bisulphite

LEONELL C. STRONG\* (Introduced by S. Bayne-Jones)

*From the Department of Anatomy, Yale University School of Medicine*

Evidence has been presented that demonstrates quite convincingly that the growth rate and fate of spontaneous carcinoma of the mammary gland in mice may be significantly influenced by heptyl aldehyde.<sup>1</sup> The effect of liquefaction (both partial and in some cases complete) together with a slower growth rate and complete disappearance of the tumor mass could be brought about by the introduction into the organism of the drug either by the diet or by subcutaneous injection (at areas remote to the spontaneous tumor). One of the limiting factors in the introduction of the material

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<sup>1</sup> Strong, L. C. *Proc. Soc. Exp. Biol. and Med.*, 1932, **30**, 386.

through the diet is the fact that the resulting mixed food becomes so bitter with the larger effective doses that the normal food habits of the mouse may be interfered with. The greatest limiting factor of the injection technic is that local ulceration is sometimes quite pronounced.

In a recent paper, it was pointed out that in order to influence malignancy the heptyl aldehyde must be used fresh. It was found that as soon as the heptyl aldehyde-normal diet mix had "aged" even for a few days the efficacy of the drug was greatly diminished. One way of "protecting" the precocious deterioration of the heptyl aldehyde was the addition to it of a small amount of methyl salicylate—that is, to approach more or less the chemical situation in the original true or natural oil of Gaultheria.<sup>2</sup> Since, however, the heptyl aldehyde undergoes oxidation readily, one may prohibit or delay, to a certain extent, this change by either of two methods: (1) the addition of a known anti-oxidant, such as salicylic aldehyde to it, or (2) the combining of it with such a chemical as sodium bisulphite. Both of these suggestions have been tried, but the results obtained on spontaneous cancer with the second method alone will be discussed in the present communication.

Heptyl aldehyde-sodium bisulphite addition product† is water soluble. It is fairly stable in the dry state, but when in solution, it comes into contact with the tissues of the body, it slowly gives up its heptyl aldehyde.

Seventy-five female mice of the Strong A strain with spontaneous tumors of the mammary gland have so far been injected with freshly

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<sup>2</sup> Strong, L. C., *Yale J Biol and Med*, 1939, 11, 207.

† The material from National Drug Company was prepared by mixing heptyl aldehyde C.P. and sodium bisulphite C.P. together in equimolecular proportions. Upon stirring the mixture became solid in from 5 to 10 minutes. It was then filtered and dried. The compound, thus formed, is of a brilliant mother-of-pearl lustre and appears to be crystalline. It contains 52.3% of heptylaldehyde. The sample from R. Auchincloss was prepared according to Cumming, Hopper and Wheeler (*Systematic Organic Chemistry: Modern Methods of Preparation and Estimation*, p. 518) from a reactive solution of  $\text{NaHSO}_3$ . Fifteen grams of  $\text{Na}_2\text{CO}_3$  were put into 100 cc  $\text{H}_2\text{O}$ .  $\text{SO}_2$  was bubbled through. This gave a white precipitate. The  $\text{SO}_2$  was passed 50 minutes when solution was cleared of white precipitate. At this time the solution did not yet smell of  $\text{SO}_2$ . After another 25 minutes the evolution of  $\text{CO}_2$  stopped and the solution smelled of  $\text{SO}_2$ . 18.8 cc heptylaldehyde (Fastman) was then added and shortly later another 18.8 cc (100% excess). White precipitate was formed quickly. It was left in the ice box overnight. The material was then filtered and the crystals were washed with a small amount of 95% alcohol. A micro sulfur determination was 13.9% theoretical 14.6%. The resulting compound is water soluble and is stable in solution at neutral pH.

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<sup>1</sup> Strong, L. C. *Proc. Soc. Exp. Biol. and Med.*, 1932, **30**, 386.

without interfering with the body weight or physical condition of the mouse

By an inspection of the chart it may be seen that there is an indirect correlation between the growth rate of spontaneous tumors and the amount of heptyl aldehyde-sodium bisulphite addition compound injected. The same large areas of liquefaction were found here as were obtained by the use of heptyl aldehyde alone. Complete disappearance of some of the spontaneous tumors (6 out of 25 in the fifth series) did occur when the level of 6 mg per day was reached. There have been no recurrences in these cases and the mice are living in normal health at the present time.

A mouse, in which a large tumor is breaking down and being eliminated, is in a critical stage. Some of them die during this process primarily by hemorrhage from the surface of the tumor. This untoward result may be prevented by (1) clipping the hair over the tumor, (2) a periodic bathing of the tumor in 70% alcohol, (3) a drainage by sterile puncture and (4) packing a delicate or thin layer of sterile cotton over a potential 'break' in the surface of the tumor. So far the largest tumor that has completely regressed in this series on the chemotherapy of cancer was one that measured 1.5 cm in longest linear diameter.

Heptyl aldehyde-sodium bisulphite addition compound has the further advantage over heptyl aldehyde alone by the fact that local ulceration is considerably reduced.

Experiments with other series of mice receiving larger daily doses of heptyl aldehyde-sodium bisulphite addition compound are being done.

prepared distilled water solutions of heptyl aldehyde-sodium bisulphite addition compound. The mice were divided into 5 groups depending upon the amount of the addition product they received daily. The first group of 5 mice received 2 mg of the material dissolved in 0.20 cc of distilled water, the second group of 5 mice, 3 mg in the same amount of water, the third group of 15 mice, 4 mg, the fourth group of 25 mice, 5 mg, and the fifth group of 25 mice 6 mg. The growth rates of the tumors in the various experimental groups are compared with that for the tumors of 120 control mice of the same strain in Chart 1.

All injections were done at sites remote to the spontaneous tumors. The variable factors involved in the tolerance to, and the physiological effect of the drug are (1) age of the mouse, (2) body weight, (3) physical condition of the mouse, (4) size of the tumor, and (5) individual peculiarities of both the mouse and certain tumors. The dosage administered to each mouse was carefully determined each day and was altered according to the above variable criteria. The idea used throughout was to inject the maximum effective dose.

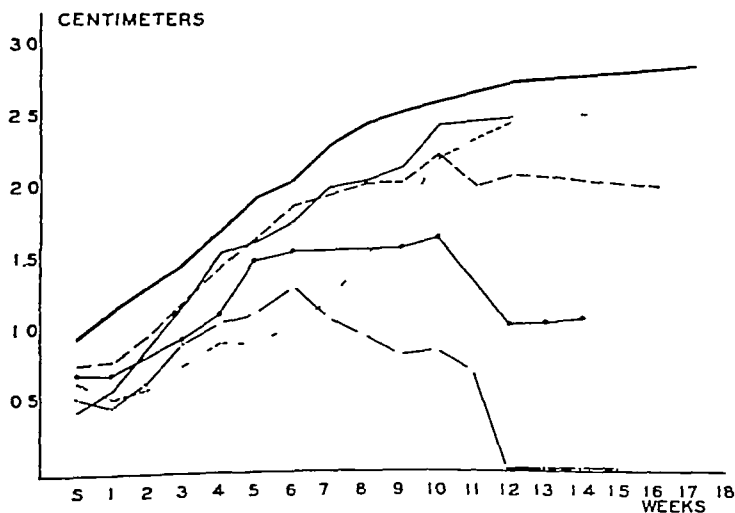


CHART 1

Chart 1 presents data obtained on the growth rate of spontaneous tumors of the mammary gland in mice on (a) 120 control mice (heavy solid line), (b) 5 mice receiving 2 mg per day heptyl aldehyde sodium bisulphite addition product (light solid line), (c) 5 mice receiving 3 mg daily of the same drug (short dash line), (d) 15 mice receiving 4 mg per day of the same drug (long dash line), (e) 25 mice getting 5 mg per day (solid ball and solid line), and (f) 25 mice getting 6 mg per day (dot and broken line).

without interfering with the body weight or physical condition of the mouse

By an inspection of the chart it may be seen that there is an indirect correlation between the growth rate of spontaneous tumors and the amount of heptyl aldehyde-sodium bisulphite addition compound injected. The same large areas of liquefaction were found here as were obtained by the use of heptyl aldehyde alone. Complete disappearance of some of the spontaneous tumors (6 out of 25 in the fifth series) did occur when the level of 6 mg per day was reached. There have been no recurrences in these cases and the mice are living in normal health at the present time.

A mouse, in which a large tumor is breaking down and being eliminated is in a critical stage. Some of them die during this process primarily by hemorrhage from the surface of the tumor. This untoward result may be prevented by (1) clipping the hair over the tumor, (2) a periodic bathing of the tumor in 70% alcohol, (3) a drainage by sterile puncture and (4) packing a delicate or thin layer of sterile cotton over a potential "break" in the surface of the tumor. So far the largest tumor that has completely regressed in this series on the chemotherapy of cancer was one that measured 1.5 cm in longest linear diameter.

Heptyl aldehyde-sodium bisulphite addition compound has the further advantage over heptyl aldehyde alone by the fact that local ulceration is considerably reduced.

Experiments with other series of mice receiving larger daily doses of heptyl aldehyde-sodium bisulphite addition compound are being done.

prepared distilled water solutions of heptyl aldehyde-sodium bisulphite addition compound. The mice were divided into 5 groups depending upon the amount of the addition product they received daily. The first group of 5 mice received 2 mg of the material dissolved in 0.20 cc of distilled water, the second group of 5 mice, 3 mg in the same amount of water, the third group of 15 mice, 4 mg, the fourth group of 25 mice, 5 mg, and the fifth group of 25 mice 6 mg. The growth rates of the tumors in the various experimental groups are compared with that for the tumors of 120 control mice of the same strain in Chart 1.

All injections were done at sites remote to the spontaneous tumors. The variable factors involved in the tolerance to, and the physiological effect of the drug are (1) age of the mouse, (2) body weight, (3) physical condition of the mouse, (4) size of the tumor, and (5) individual peculiarities of both the mouse and certain tumors. The dosage administered to each mouse was carefully determined each day and was altered according to the above variable criteria. The idea used throughout was to inject the maximum effective dose

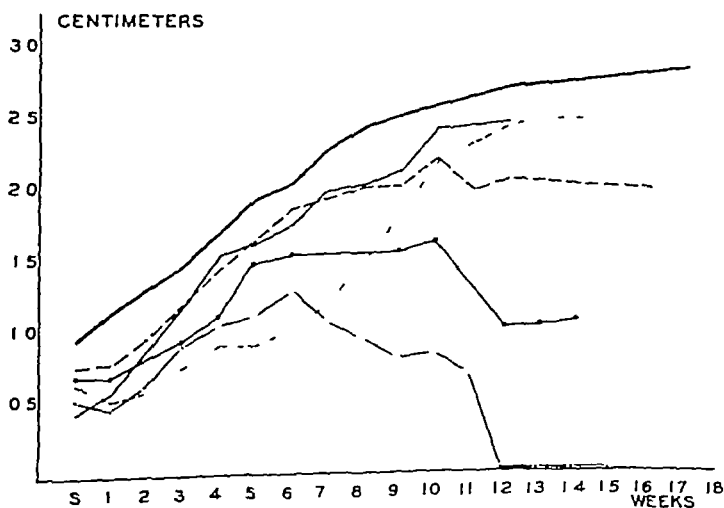


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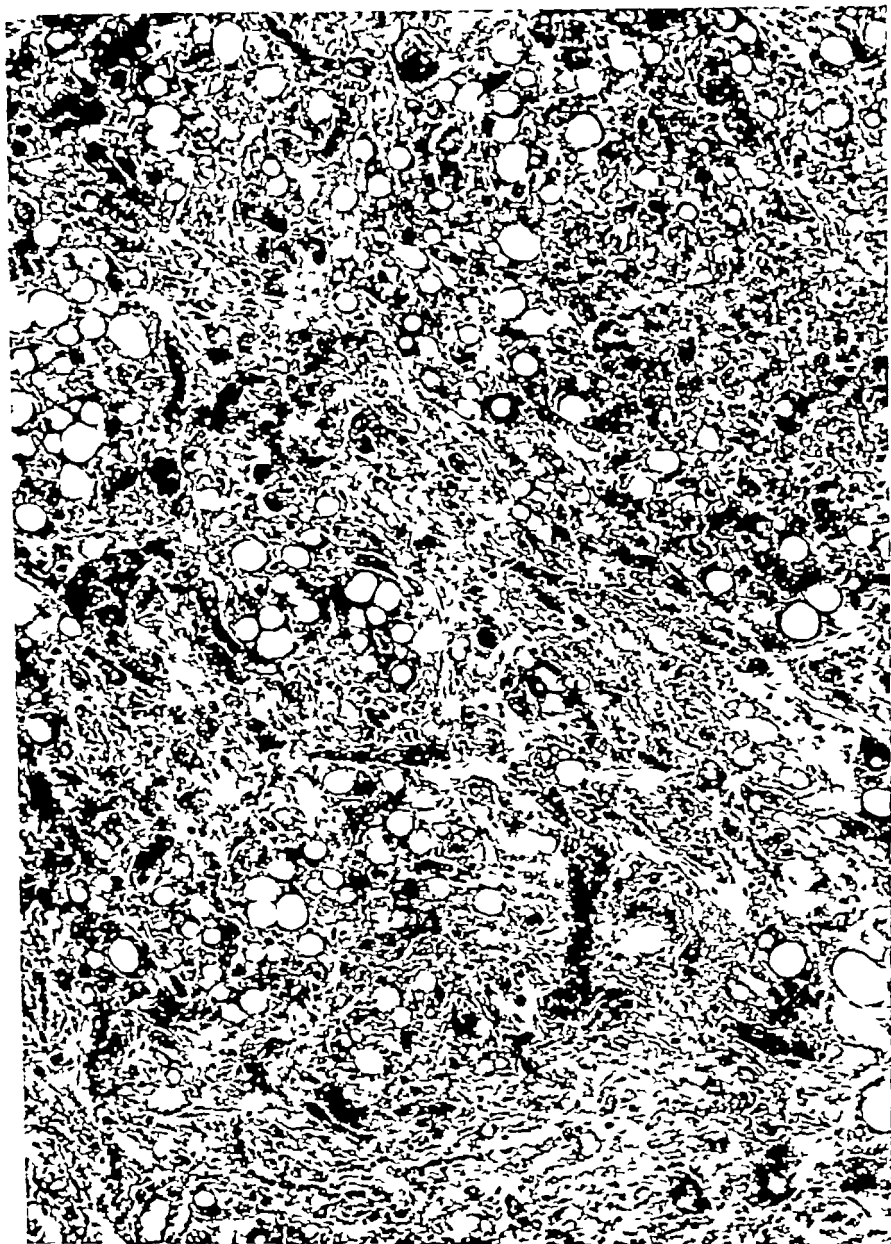


FIG 1

Microphotograph of sections stained with Mallory's aniline blue collagen stain. At a magnification at about 170X the uniform distribution of fibrous tissue, fat and degenerating liver cells can be seen. Many large sinuses are engorged with blood.



## Production of Cirrhosis of the Liver of the Normal Dog by High Fat Diets \*

I L CHAIKOFF AND C L CONNOR

*From the Divisions of Physiology and Pathology University of California Medical School, Berkeley and San Francisco*

During the course of a study on the maintenance of the completely depancreatized dog it was observed that cirrhosis of the liver developed in 8 of the 16 animals that had been kept alive with insulin for periods between 2 6 and 5 5 years<sup>1</sup> Infection and obstruction of the extrahepatic bile passages were ruled out as factors that might have induced the scarring of the liver The animals received a diet containing lean meat, sucrose bone ash and vitamin supplements and despite its completeness with respect to calories, proteins vitamins and salts it has been demonstrated repeatedly that this diet induces fatty livers in depancreatized dogs Although it was recognized that the absence *per se* of the pancreas might be involved, it was nevertheless suggested that an important causative factor in the production of the hepatic fibrosis was the excessive deposition of fat which appeared in the liver during the first few months after pancreatectomy and remained there for long periods

In view of the importance of the above findings, it seemed highly desirable to determine whether cirrhosis could be induced in the fatty liver of the normal dog The observations recorded below establish for the first time that the continued presence of excessively high amounts of fat in the liver for several months stimulates hepatic fibrosis even in normal dogs to the extent that it may be called a true cirrhosis, and cause the death of the animals

*Experimental* Well nourished dogs were selected for use in this experiment Throughout the periods of observation they were maintained on a high fat diet that contained 10 g of lard and 7 g of lean meat per kilo per day They were fed twice daily by stomach tube when they would not eat at 7 00 a m and at 2 30 p m Each animal also received once daily 1 g of Cowgill's salt mixture<sup>2</sup> 1-5 g of bone ash and vitamin supplements in the form of cod liver oil and a rice bran concentrate

Four dogs died after being maintained on this diet for 138 246

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\* Aided by a grant from the Christine Breon Fund for Medical Research

1 Chaikoff, I L, Connor, C L, and Biskind, G R, *Am J Path*, 1938, 14, 101

2 Cowgill, G R, *J Biol Chem* 1923, 56, 725

298, and 386 days. The livers of 3 of these animals showed a severe diffuse fibrosis and in all of them a severe fatty infiltration was observed. A typical protocol follows.

Dog F41 was placed on the high fat diet on November 29, 1938, and died December 20, 1939. Its weight at the start was 11.2 kg and at death 12.8 kg. It attained a maximum weight of 16.5 kg during the course of the experiment. The liver weighed 695 g. It was uniformly green in color, firm and rubbery in consistency. It cut with resistance and was lobulated on the surface.

Microscopically (Figs 1 and 2) the liver showed marked fibrosis and a fatty infiltration. The liver cords were broken up into fragments and in no place had the original pattern of the liver been retained. Fragments of cells were found between proliferating strands of connective tissue that were growing in all directions but had no particular distribution with respect to portal areas or central veins. Blood sinuses were engorged and appeared like fibrous walled blood vessels rather than sinusoids. Some bile duct proliferation had taken place but their duct cells were difficult to recognize because they, too, were enmeshed in connective tissue. Many plugs of inspissated bile were present in large and small masses.

It is of interest to note that the diffuse character of the fibrosis reported here distinguishes this type of cirrhosis from that previously described for dogs receiving alcohol,<sup>3</sup> although the outstanding anatomical change preceding the fibrosis in both instances, as well as in the depancreatized dogs, was severe fatty infiltration of the liver.

11288 P

### Immediate Generalized Skin Reactions in Hypersensitive Guinea Pigs \*

J. L. JACOBS (Introduced by V. Menkin)

*From the Department of Pathology and Bacteriology, Tufts College Medical School,  
Boston, Mass*

Immediate generalized skin-reactions following contact with or ingestion of allergens have been observed in humans but not hitherto in animals, although Dienes and Simon<sup>1</sup> reported a generalized

<sup>3</sup> Connor, C. L., and Chaikoff, I. L., *Proc. Soc. Exp. Biol. and Med.*, 1938, **30**, 356.

\* This investigation was aided by a grant from the Charlton Research Fund of the Tufts College Medical School.

<sup>1</sup> Dienes, L. and Simon, F. A., *J. Immunol.*, 1935, **28**, 321.

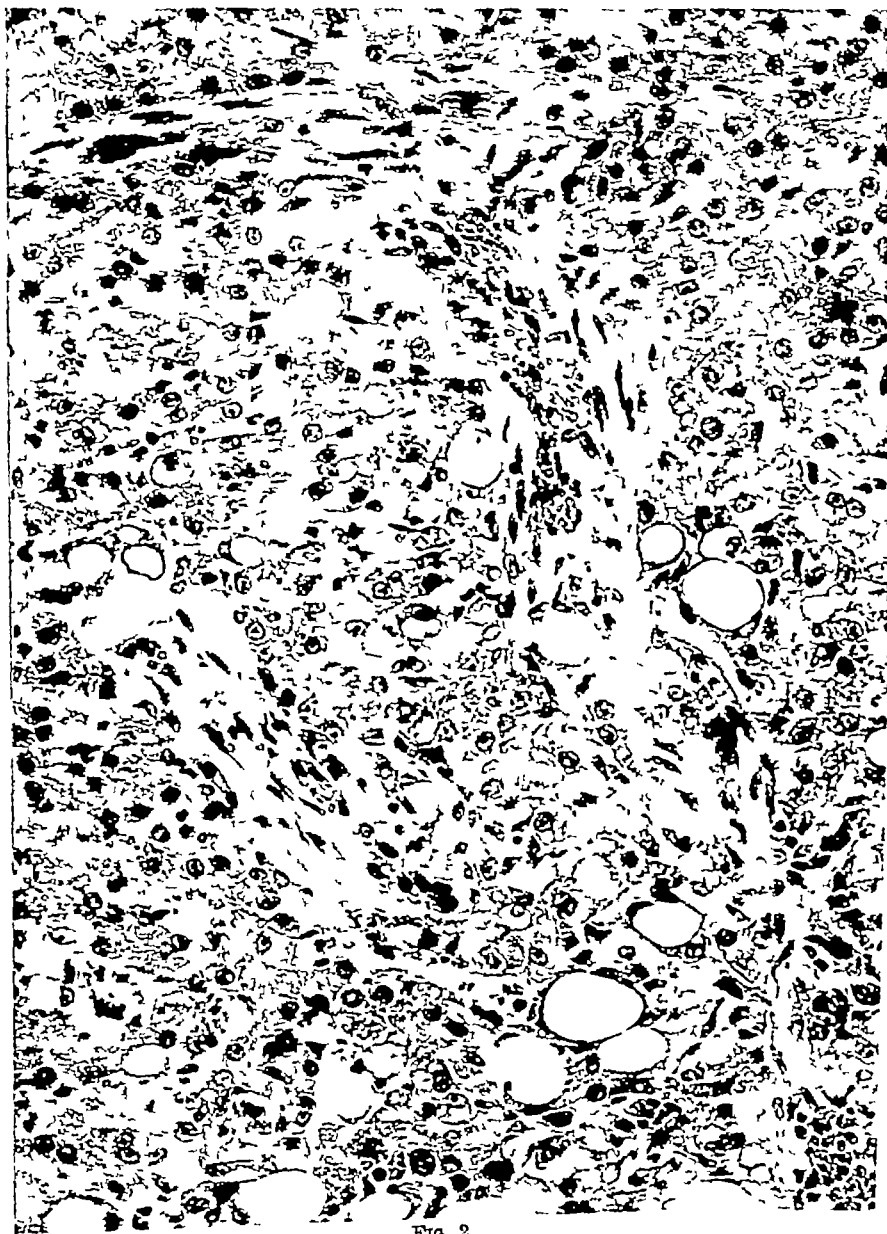


FIG 2

Microphotograph of sections stained with Mallory's aniline blue collagen stain. At about 350X, more details of the proliferating fibrous tissue can be seen. In Figs 1 and 2 the lack of portal or perlobular distribution of the process is evident.

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<sup>1</sup> Dienes, L., and Simon, F. A., *J. Immunol.*, 1935, **28**, 321.

delayed reaction in guinea pigs treated with human serum. No observations of generalized skin-reactions in animals, due to simple chemical substances, have been noted in the literature. It seemed of interest, therefore, to report recent observations of such effects in a number of guinea pigs sensitized to citraconic anhydride, together with the sensitization of guinea pigs to new anhydrides having similar properties.

The animals were rendered hypersensitive to citraconic anhydride by a method previously described<sup>2</sup> and were tested by scratching the skin through a 25% dioxane solution of the same substance, usually at intervals of 2 weeks.

It was regularly observed that the more hypersensitive the guinea pig the quicker the onset of the reaction. Animals developing generalized reactions showed within a very few minutes a large local wheal and erythema surrounding the scratch, often several centimeters in diameter. At the same time in both contiguous and remote parts of the skin some pigs showed a diffuse pinkishness apparently accompanied by slight thickening, while others exhibited discrete, urticaria-like wheals with distinct elevation and pinkness. Often some of the old injection-sites on the back flared up at the same time, with marked wheal-formation. The erythema reached a maximum 20 to 30 minutes after the test, following which it gradually faded. Edema associated with reactivated sites and other lesions distant from the original scratch increased for about 2 hours and then also diminished. By the next day all such subsidiary reactions had completely disappeared. In striking, possibly significant contrast to this, the reaction in the vicinity of the scratch after receding to a minimal color and edema in 4 to 5 hours, gradually increased in pinkishness and induration to a second maximum reached about 24 hours after the test. This secondary or delayed reaction at the original site slowly disappeared in the course of several more days. At no time were there any definite symptoms of anaphylaxis although coughs and shivering were occasionally observed. The mechanism of these effects requires further study.

These phenomena invite comparison with the occasional severe generalized skin-reactions from drugs observed in humans. Practically all guinea pigs treated with citraconic anhydride become hypersensitive but in our experiments only 6 out of 61, about 10%, gave generalized reactions. Other pigs less strongly sensitized, however, produced similar but less intense reactions, the subsidiary

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<sup>2</sup> Jacobs, J. L., Golden, T. S. and Kelley, J. J., *Proc. Soc. Exp. Biol. and Med.*, 1940, **43**, 74.

lesions being fewer in number and not present on areas far from the scratch. Unsensitized control animals were quite negative.

In small-scale experiments with 2 new substances, maleic and acetic anhydrides, using the same technic as with citraconic anhydride,<sup>2</sup> extensive immediate wheal-and-erythema reactions were produced, with subsidiary reactions in some cases. With maleic anhydride such isolated reactions appeared many centimeters from the site of scratch. It would seem profitable to examine, on a larger scale, the capacity of these compounds to produce generalized reactions.

Excellent sensitization-effects have been produced by the same method with a third substance, propionic anhydride, using patch-tests with a 25% dioxane solution. Scratch-tests, however, did not yield definite wheal-and-erythema reactions, which is interesting in view of the close chemical relationship between this compound and acetic anhydride.

## 11289 P

### Solubility of Fluorosed Enamel and Dentine

J F VOLKER \* (Introduced by H C Hodge)

*From the Department of Biochemistry and Pharmacology, University of Rochester School of Medicine and Dentistry, Rochester, N Y*

The reduction of caries incidence in humans and rats ingesting fluorine may be the result of a decreased acid solubility due to the incorporation of fluorine in the dental tissues. Support for this belief is apparent in the finding of more fluorine in caries-resistant than carious enamel and dentine.<sup>1</sup> If this assumption is correct, we might expect the greatest reduction in acid solubility in the enamel and dentin from mottled teeth since it has been shown that these teeth contain the greatest amounts of fluorine.<sup>2</sup>

The acid solubility of normal and fluorosed rat teeth was studied. The upper incisor teeth were used in all cases. The fluorosed human teeth were obtained from areas where dental fluorosis is endemic and showed a mild degree of "mottling." The fluorosed rat incisors represented were of 2 grades, (1) *mild*, produced by a diet

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\* Carnegie Dental Fellow

<sup>1</sup> Armstrong, W D, and Brekhus, P J, *J Dent Res*, 1938, **17**, 339

<sup>2</sup> Armstrong, W D, and Brekhus, P J, *J Dent Res*, 1938, **17**, 27

containing 20 p p m of dietary fluorine and (2) *severe*, produced by a diet containing 300 p p m of fluorine

The teeth to be tested were powdered to pass a 100 mesh screen Separation and purification of the enamel and dentin was accomplished by the centrifugation-flotation method<sup>3</sup> Solubilities of the fluorosed and normal samples were compared by measuring the weight losses of duplicate 50 mg samples after definite time intervals in 20 cc amounts of 2 M acetic acid/sodium acetate buffer at pH 4.0<sup>4</sup> Whenever sufficient amounts of the experimental materials were available, measurements were recorded at varying intervals up to and including one hour Where the supply of experimental material was limited, quadruplicate solubility determinations were made at the end of one hour

**Results** The experimental curves for normal and fluorosed rat (300 p p m) and normal human enamels and dentins may be seen in Fig 1 Each point represents an average of 3 determinations The solubilities of normal human and rat enamel are similar, as are those

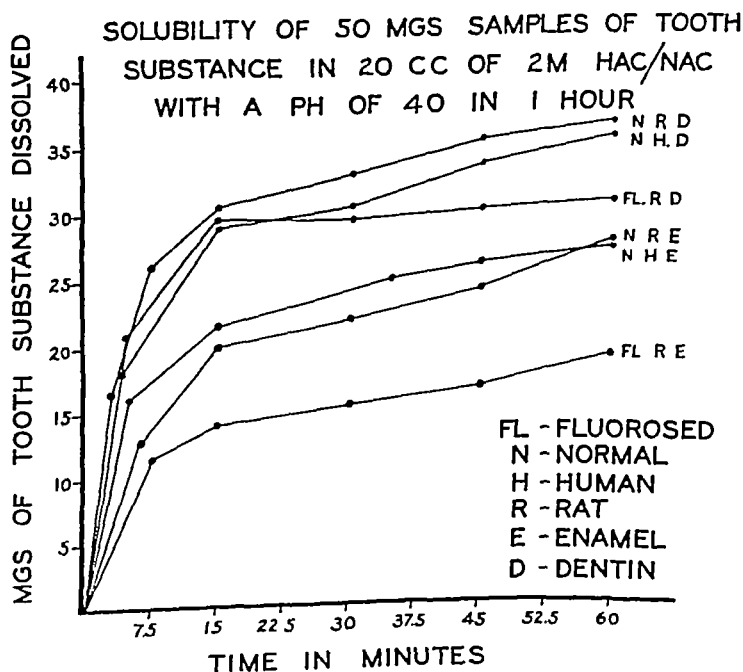


FIG 1

<sup>3</sup> Manly, R S, and Hodge, H. C, *J Dent Res*, 1939, 18, 133

<sup>4</sup> Volker, J, Thesis, University of Rochester, Rochester, N Y, 1939

of normal human and rat dentin. In each case the dentin is considerably more soluble than the enamel. Severely fluorosed rat (300 ppm) enamel and dentin have significantly reduced acid solubilities. Both the enamel and dentin solubility values of the mildly fluorosed rat (20 ppm) tissues are comparable to those found in the control tissues (27.1 mg of enamel and 37.8 mg of dentin being lost in one hour). The weight loss of the mildly fluorosed human enamel samples for a one-hour period (26.9 mg) closely approximates that of the normal human enamel (26.6 mg) but the fluorosed human dentin shows a decreased solubility (32.1 mg) when compared with normal human dentin (37.8).

*Comment* The findings indicate that the fluorine content of the tooth modifies its solubility. This is not surprising since the presence of fluoride decreases the solubility of related calcium phosphate.<sup>5</sup> Fluorosed rat enamel (300 ppm) containing 125% fluorine was less soluble than normal rat enamel containing 0.08% fluorine.<sup>6</sup> Parallels are also noted with fluorosed rat dentin (300 ppm) having 25% fluorine and normal rat dentin having only 0.1% fluorine<sup>7</sup> and with fluorosed human dentin having 0.7% fluorine<sup>2</sup> and normal human dentin having only 0.17% fluorine.<sup>2</sup> The inability to demonstrate any reduction in solubility of mildly fluorosed human enamel and rat enamel and dentin (20 ppm) may be explained by the low fluorine content of these tissues.

*Conclusion* The presence of fluorine in large amounts may decrease the solubility of the dental hard tissues. Small amounts of fluorine show no demonstrable reduction in enamel solubility. It seems doubtful that the amounts of fluorine present in slightly fluorosed teeth is sufficient to alter their acid solubility.

The kindness of Drs. G. J. Cox, H. C. Hodge and S. B. Finn in supplying the rat teeth and Dr. W. Armstrong in supplying the human teeth used in this study is acknowledged.

<sup>5</sup> MacIntire, W. H., and Hammond, J. W., *Ind. and Eng. Chem.*, 1930, **30**, 160.

<sup>6</sup> Hodge, H. C., Luce Clausen, E. M., and Brown, E. F., *J. Nutrition*, 1939, **17**, 333.

<sup>7</sup> Bowes, J. H., and Murry, M. M., *Brit. Dent. J.* 1936, **60**, 556.



# Preparation and Immunological Properties of Acid-Denatured Egg Albumin \*

CATHERINE F C MACPHERSON AND MICHAEL HEIDELBERGER

*From the Departments of Biochemistry and Medicine, College of Physicians and Surgeons, Columbia University, and the Presbyterian Hospital, New York City*

Aqueous solutions of 3 times recrystallized Ea were allowed to stand at room temperature for 1 to 2 days with HCl between pH 1.5 and 2.3. Depending upon the acidity from 25 to 75% of the Ea was denatured. The DnEa was precipitated by adding NaOH until the isoelectric range pH 5.2 to 5.4, was reached. Three additional reprecipitations usually sufficed to remove unchanged Ea. The pre-

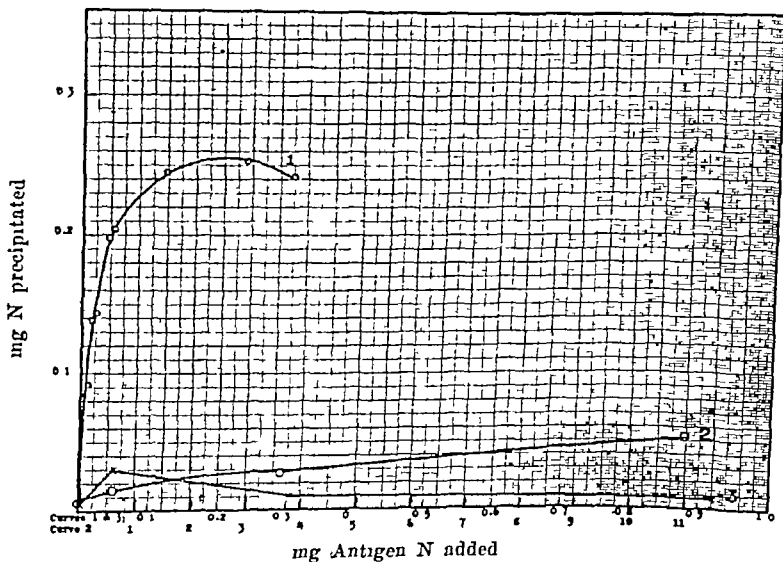


FIG. 1

Precipitation of Anti DnEa and Anti Ea Sera by DnEa and Ea

Curve 1—N precipitated from anti DnEa by DnEa. The Ea anti Ea curve is similar.

Curve 2—N precipitated from anti DnEa by Ea.

Curve 3—N precipitated from anti Ea by DnEa. Supernatants in this series remained opalescent.

Sera compared at approximately equal antibody-content.

\* Egg albumin and acid denatured egg albumin are subsequently referred to as Ea and DnEa respectively. This note is from a dissertation to be submitted by Catherine MacPherson in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Faculty of Pure Science, Columbia University.

precipitate was dispersed in water dissolved at pH 7, and allowed to stand in the cold for 4 to 5 days after which it was more stable to physiological concentrations of NaCl. Reversion to Ea did not take place.

Three preparations which differed in appearance and solubility in water at pH 7 were found to remove, per mg of DnEa N, the same amount of N from a calibrated anti-DnEa serum.

The reaction between DnEa and its homologous antibody is similar to that between Ea and anti-Ea<sup>1</sup>. The cross-reactions between anti-Ea and DnEa and between anti-DnEa and Ea differ greatly from both homologous reactions and from each other, as shown in Fig. 1.

A study of the chemical and physical properties of Ea denatured in various ways and a correlation of these properties with the immunological findings is now in progress. Previous work in this field appears to have been confined to a preliminary note by Wu, TenBroeck, and Li,<sup>2</sup> the studies of Flosdorff and Chambers<sup>3</sup> and Blumenthal,<sup>4</sup> and the work of Porter and Pappenheimer,<sup>5</sup> and Landsteiner and Rothen<sup>6</sup> on surface-films.

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<sup>1</sup> Heidelberger, M., and Kendall, F. E., *J. Exp. Med.*, 1935, **62**, 697.

<sup>2</sup> Wu, H., Ten Broeck, C., and Li, C. P., *Proc. Soc. Exp. Biol. and Med.*, 1926 **7**, **24**, 472.

<sup>3</sup> Flosdorff, E. W., and Chambers, L. H., *J. Immunol.*, 1935, **28**, 297.

<sup>4</sup> Blumenthal, D., *J. Biol. Chem.*, 1936, **118**, 433.

<sup>5</sup> Porter, E. F., and Pappenheimer, A. M., Jr., *J. Exp. Med.*, 1939, **69**, 755.

<sup>6</sup> Landsteiner, K., and Rothen, A., *Science*, 1939, **90**, 65.

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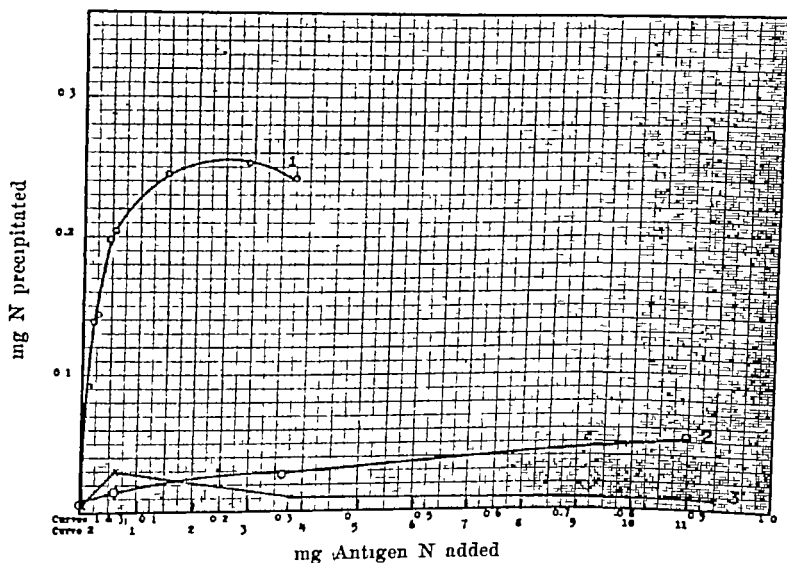


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This is spun 15 minutes at 15,000 g and the supernatant fluid diluted to 30 cc. From this volume the protein is sedimented at 67,000 g for 30 minutes. The 2 pellets are dissolved in 1.5 to 3 cc of Ringer's solution pH 9.0 depending on yield.

The character of the product with respect to homogeneity can be judged by the photometric tracing in Fig. 1. Practically all of the normal chick component<sup>2</sup> is eliminated in the first ultracentrifugal cycle and no evidence of it has been observed after the second cycle. Similar loss of colloid occurs in the process of filtration and in the first and second ultracentrifugal cycles.

Only slight loss of infectivity occurs in filtration, and no evidence is seen of damage to the protein by short exposure to pH 6.5. Precipitated at pH 6.5 the purified protein redissolves to its initial homogeneity and infectivity. Acidification facilitates sedimentation and separation of the protein from the normal chick component in the first relatively low speed cycle and greatly increases the final yield of the homogeneous product, ca 0.4 mg per gram of diseased embryo.

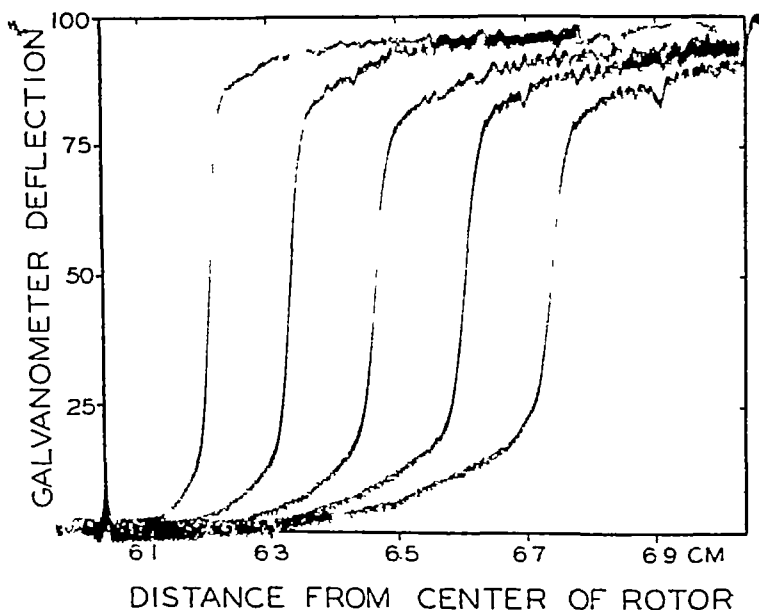


FIG. 1

Photometer curves from sedimentation diagrams of a typical preparation of Eastern strain equine encephalomyelitis virus protein purified by the method described. The protein concentration was 1.8 mg per cc, the interval between curves 5 minutes and the ultracentrifugal field 17,000 g.

## Improved Sequence for Rapid Consistent Purification of Equine Encephalomyelitis Virus Protein \*

A R TAYLOR, D G SHARP, HAROLD FINKELSTEIN, DOROTHY BEARD AND J W BEARD

*From the Department of Surgery, Duke University School of Medicine,  
Durham, N C*

The procedure described here for purification of equine encephalomyelitis virus protein<sup>1</sup> yields consistently a product of high molecular homogeneity. Advantage is taken chiefly of the following principles: (1) prolonged extraction to aid in eliminating the normal chick tissue component,<sup>2</sup> (2) extraction, fractionation and solution of the protein in a balanced salt solution, mammalian Ringer,<sup>3</sup> instead of 0.9% NaCl or buffer salt solutions, (3) filtration of crude extracts with celite to remove mucoid and colloid materials, and (4) aggregation or partial precipitation of the protein in slightly acid medium prior to the first ultracentrifugal cycle.

Diseased embryo tissue ground in the cold in the usual way,<sup>2</sup> is suspended in 4 times its volume of normal Ringer's solution made to pH 9.0 with  $\text{NH}_4\text{OH}$  without buffer salts. After extraction at 5°C for 72 to 96 hours, gross material is eliminated in the angle centrifuge. To each 100 cc of the turbid supernatant fluid, 5 g of No. 512 Celite Filter Aid (Johns-Manville Co., N. Y.) are added and the suspension is filtered with suction through a 1 to 2 mm mat of No. 503 Celite. Standard celite then added to the filtrate, 2 g per 100 cc, is filtered off through a mat also of standard celite. Sometimes the latter step is repeated to obtain an entirely clear filtrate.

The filtrate is acidified to pH 6.5 with 0.2 N HCl, and 120 cc of it distributed immediately in 8 collodion tubes is spun at 17,000 g for 45 minutes. The pellets are taken up in 30 cc of Ringer's solution. In 2 tubes, the solution is spun at 17,000 g for 5 minutes. The supernatant fluid diluted to 60 cc is ultracentrifuged 30 minutes at 67,000 g and the resulting pellets are taken up in 15.0 cc of Ringer fluid.

\* This work was aided by the Dorothy Beard Research Fund and by a grant from Lederle Laboratories, Pearl River, New York.

<sup>1</sup> Taylor, A. R., Sharp, D. G., Finkelstein, H., and Beard, J. W. *Proc. Soc. Exp. Biol. and Med.*, 1939, **42**, 462.

<sup>2</sup> Sharp, D. G., Taylor, A. R., Finkelstein, H., and Beard, J. W., *Proc. Soc. Exp. Biol. and Med.*, 1939, **42**, 459.

<sup>3</sup> Bayliss, W. M., *Principles of Physiology*, Longmans, Greep and Co., Ltd., London, 4th ed., page 211.

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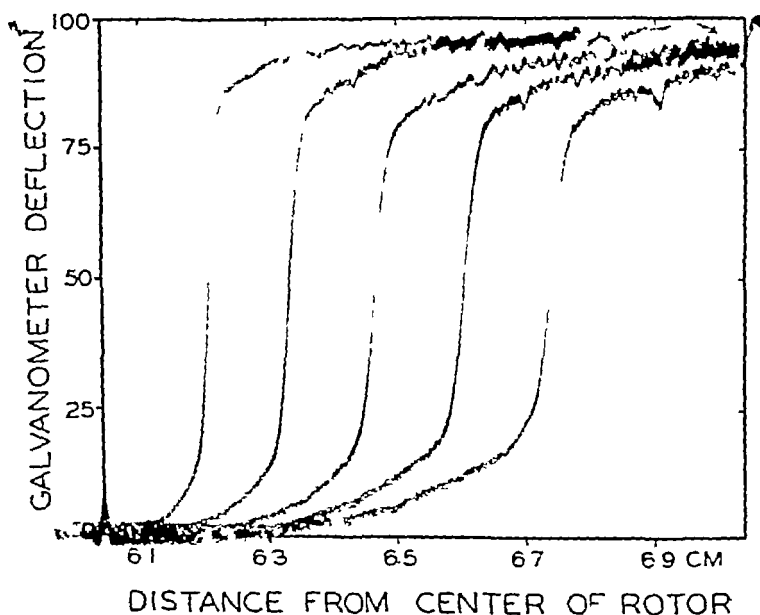


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Durham, N C*

The procedure described here for purification of equine encephalomyelitis virus protein<sup>1</sup> yields consistently a product of high molecular homogeneity. Advantage is taken chiefly of the following principles: (1) prolonged extraction to aid in eliminating the normal chick tissue component,<sup>2</sup> (2) extraction, fractionation and solution of the protein in a balanced salt solution, mammalian Ringer,<sup>3</sup> instead of 0.9% NaCl or buffer salt solutions, (3) filtration of crude extracts with celite to remove mucoid and colloid materials, and (4) aggregation or partial precipitation of the protein in slightly acid medium prior to the first ultracentrifugal cycle.

Diseased embryo tissue ground in the cold in the usual way,<sup>2</sup> is suspended in 4 times its volume of normal Ringer's solution made to pH 9.0 with  $\text{NH}_4\text{OH}$  without buffer salts. After extraction at 5°C for 72 to 96 hours, gross material is eliminated in the angle centrifuge. To each 100 cc of the turbid supernatant fluid, 5 g of No. 512 Celite Filter Aid (Johns-Manville Co., N. Y.) are added and the suspension is filtered with suction through a 1 to 2 mm mat of No. 503 Celite. Standard celite then added to the filtrate, 2 g per 100 cc, is filtered off through a mat also of standard celite. Sometimes the latter step is repeated to obtain an entirely clear filtrate.

The filtrate is acidified to pH 6.5 with 0.2 N HCl, and 120 cc of it distributed immediately in 8 collodion tubes is spun at 17,000 g for 45 minutes. The pellets are taken up in 30 cc of Ringer's solution. In 2 tubes, the solution is spun at 17,000 g for 5 minutes. The supernatant fluid diluted to 60 cc is ultracentrifuged 30 minutes at 67,000 g and the resulting pellets are taken up in 150 cc of Ringer fluid.

\* This work was aided by the Dorothy Beard Research Fund and by a grant from Lederle Laboratories, Pearl River, New York.

<sup>1</sup> Taylor, A. R., Sharp, D. G., Finkelstein, H., and Beard, J. W. *Proc. Soc. Exp. Biol. and Med.*, 1939, **42**, 462.

<sup>2</sup> Sharp, D. G., Taylor, A. R., Finkelstein, H., and Beard, J. W. *Proc. Soc. Exp. Biol. and Med.*, 1939, **42**, 459.

<sup>3</sup> Bayliss, W. M., *Principles of Physiology*, Longmans, Greep and Co., Ltd., London, 4th ed., page 211.

This is spun 15 minutes at 15,000 g and the supernatant fluid diluted to 30 cc. From this volume the protein is sedimented at 67,000 g for 30 minutes. The 2 pellets are dissolved in 1.5 to 3 cc of Ringer's solution pH 9.0 depending on yield.

The character of the product with respect to homogeneity can be judged by the photometric tracing in Fig. 1. Practically all of the normal chick component<sup>2</sup> is eliminated in the first ultracentrifugal cycle and no evidence of it has been observed after the second cycle. Similar loss of colloid occurs in the process of filtration and in the first and second ultracentrifugal cycles.

Only slight loss of infectivity occurs in filtration, and no evidence is seen of damage to the protein by short exposure to pH 6.5. Precipitated at pH 6.5 the purified protein redissolves to its initial homogeneity and infectivity. Acidification facilitates sedimentation and separation of the protein from the normal chick component in the first relatively low speed cycle and greatly increases the final yield of the homogeneous product, ca 0.4 mg per gram of diseased embryo.

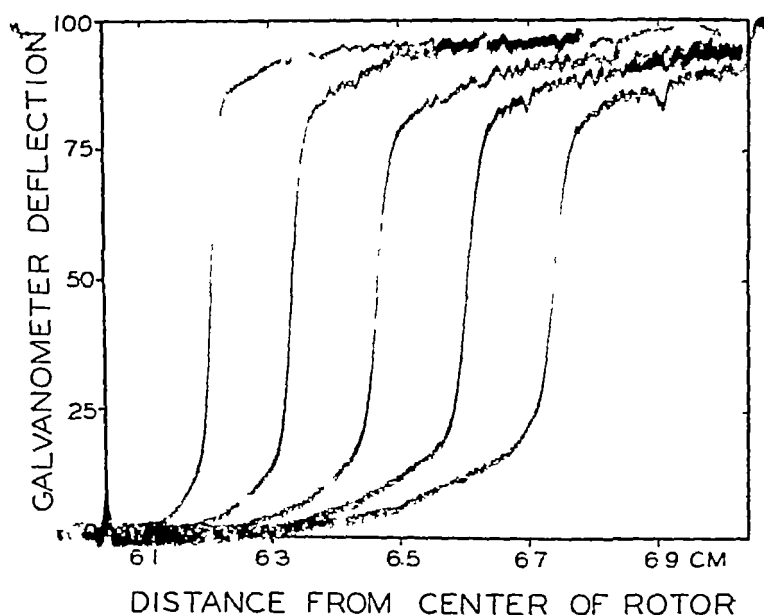


FIG 1

Photometer curves from sedimentation diagrams of a typical preparation of Eastern strain equine encephalomyelitis virus protein purified by the method described. The protein concentration was 1.8 mg per cc, the interval between curves 5 minutes and the ultracentrifugal field 17,000 g.



tissue In Ringer's solution, at a protein concentration of 1 to 1.5 mg per cc, infectivity does not diminish within 7 days at 5°C and the sedimentation pattern is unchanged for periods of several weeks In 0.9% NaCl or Ringer-buffer salt solution ( $\text{NH}_4\text{OH-NH}_4\text{Cl}$ , 0.05 M), however the protein loses much infectivity in 96 hours In the latter solution the boundary may remain intact for 3 weeks when infectivity has diminished 6 decimal dilutions or more

## 11292 P

### Immunization with Non-Infectious Formalin Derivative of Purified Equine Encephalomyelitis Virus Protein \*

D. G. SHARP, A. R. TAYLOR, HAROLD FINKELSTEIN, DOROTHY BEARD AND J. W. BEARD

*From the Department of Surgery, Duke University School of Medicine, Durham, N. C.*

Previous ultracentrifugal studies<sup>1</sup> of crude equine encephalomyelitis chick vaccines<sup>2</sup> have failed to yield definite evidence of the character of the immunizing principle Information concerning its probable nature has been sought in the present work by study of the purified virus protein treated with formaldehyde

Purified protein (Eastern strain),<sup>3</sup> dissolved in Ringer's solution, pH 8-9, 2.0 mg per cc, was treated with various concentrations of  $\text{CH}_2\text{O}$  Before exposure to  $\text{CH}_2\text{O}$ , one m. u.<sup>4</sup> of protein was  $10^{-13.5}$  g When the concentration of  $\text{CH}_2\text{O}$  was less than 0.01 M, inactivation was not always complete in 2 weeks, and tests for immunizing capacity were not made With other concentrations of  $\text{CH}_2\text{O}$  used, inactivation was complete in the time shown in Table I, as judged by the failure of  $10^{-5.5}$  g of protein to infect mice

The effect of  $\text{CH}_2\text{O}$  on the protein molecules was somewhat similar

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<sup>1</sup> Taylor, A. R., Sharp, D. G., Finkelstein, H., and Beard, J. W., *Proc. Soc. Exp. Biol. and Med.*, 1939, **42**, 462

<sup>2</sup> Beard, J. W., Finkelstein, H., Sealy, W. C., and Wyckoff, R. W. G., *Science*, 1938, **87**, 490

<sup>3</sup> Taylor, A. R., Sharp, D. G., Finkelstein, H., Beard, D., and Beard, J. W., *Proc. Soc. Exp. Biol. and Med.*, 1940, **43**, 648

<sup>4</sup> Cox, H. R., and Olitsky, P. K., *J. Exp. Med.*, 1936, **63**, 745

TABLE I  
Immunization of Guinea Pigs with the Non Infectious Formaldehyde treated  
Equine Encephalomyelitis Virus Protein

CH <sub>2</sub> O molar	Inactivation time, days	pH	Total protein* per guinea pig, mg	Test for immunity†	
				Vaccinated	Control
01	7	7.0	125	3/3‡	0/5
			250	6/6	"
02	4	6.5	125	4/4	"
			250	5/6	"
04	3	6.3	125	3/4	"
			250	4/6	"

\*Given subcutaneously in 2 equal doses in 0.1 cc at interval of 1 week.

†500 to 1,000 mouse infectious units of virus given intracerebrally

‡Numerator = survivals, denominator = No. of test guinea pigs

to its action on tobacco mosaic virus protein<sup>5</sup>. With CH<sub>2</sub>O 0.01 M or less, the protein remained in solution but lost slightly in homogeneity, showing a slightly diffuse boundary with a sedimentation pattern similar to that of the untreated protein<sup>3</sup> persisting with little change for more than 3 weeks. In 0.02 M and higher concentrations of CH<sub>2</sub>O, the protein rapidly became quite inhomogeneous and increasingly insoluble losing the definite boundary in 2-4 days. In no instance was there evidence of molecular disruption to small soluble protein fragments of uniform size, nor was there evidence of the component  $S_{20}^0 = \text{ca } 60 \times 10^{-13} \text{ cm sec}^{-1} \text{ dynes}^{-1}$  previously described<sup>6</sup>.

The non-infectious soluble though slightly inhomogeneous protein derivative with 0.01 M CH<sub>2</sub>O has immunized all guinea pigs receiving it as shown in the typical experiment in Table I. The more inhomogeneous fragments resulting from 0.02 M and higher concentrations of CH<sub>2</sub>O definitely immunize less well.

The results show that the immunizing material derived with CH<sub>2</sub>O from the pure protein consists of an inhomogeneous group of degradation products nearly the size of the intact protein or somewhat smaller. With time, as the CH<sub>2</sub>O reacts with the protein there is a progressive increase in inhomogeneity and insolubility of the products when the pH is allowed to drop from the initial value of 8-9 to pH 7.0 to 6.3 as is shown in Table I. It is probable that the immunizing material derived with CH<sub>2</sub>O from the virus in crude vaccines is of the same nature and this probability has been empha-

<sup>5</sup> Wickeff, R. W. G., Biscoe, J., and Stanley, W. M., *J. Biol. Chem.*, 1937, **117**, 57.

<sup>6</sup> Beard, J. W., Finkelstein, H., Sealy, W. C., and Wickeff, R. W. G., *Science*, 1938, **87**, 89.

tissue In Ringer's solution, at a protein concentration of 1 to 1.5 mg per cc, infectivity does not diminish within 7 days at 5°C and the sedimentation pattern is unchanged for periods of several weeks. In 0.9% NaCl or Ringer-buffer salt solution ( $\text{NH}_4\text{OH-NH}_4\text{Cl}$ , 0.05 M), however the protein loses much infectivity in 96 hours. In the latter solution the boundary may remain intact for 3 weeks when infectivity has diminished 6 decimal dilutions or more.

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Purified protein (Eastern strain),<sup>3</sup> dissolved in Ringer's solution, pH 8-9, 2.0 mg per cc, was treated with various concentrations of  $\text{CH}_2\text{O}$ . Before exposure to  $\text{CH}_2\text{O}$ , one m i u<sup>4</sup> of protein was  $10^{-13.5}$  g. When the concentration of  $\text{CH}_2\text{O}$  was less than 0.01 M, inactivation was not always complete in 2 weeks, and tests for immunizing capacity were not made. With other concentrations of  $\text{CH}_2\text{O}$  used, inactivation was complete in the time shown in Table I, as judged by the failure of  $10^{-5.5}$  g of protein to infect mice.

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<sup>2</sup> Beard, J. W., Finkelstein, H., Sealy, W. C., and Wyckoff, R. W. G., *Science*, 1938, **87**, 490.

<sup>3</sup> Taylor, A. R., Sharp, D. G., Finkelstein, H., Beard, D., and Beard, J. W., *Proc. Soc. Exp. Biol. and Med.*, 1940, **43**, 648.

<sup>4</sup> Cox, H. R., and Ohitsky, P. K., *J. Exp. Med.*, 1936, **63**, 745.

viously<sup>9</sup> an activity ratio of 1/1000 and even of 1/4000, when an especially purified sample of synthetic phthocol was used.

As already mentioned, methylnaphthoquinone in an aqueous medium is being employed clinically for intravenous medication. Aqueous or physiologic salt solutions containing 1 mg in 10 ml appear to be stable when kept in a refrigerator, and methylnaphthohydroquinone is even more soluble (3 to 4 times) in water. Recently Dann<sup>7</sup> found that oil solutions are roughly 3 times as active as aqueous ones. Our assays (Table I) show that methylnaphthoquinone, when administered orally, is about twice as potent in water as in oil, and confirm our previous observation<sup>8</sup> that the oil, used as a medium for administering vitamin K, interferes to a noticeable degree with the potency. Intravenously the aqueous solution is about as active as the oil solution orally.

The sodium salt of 2-methyl-1,4-naphthohydroquinone disulfuric acid was synthesized by Fieser and Fry<sup>6</sup> who stated that it showed antihemorrhagic activity at a level of 2%. We found (Table I) that this compound has a potency of 1 unit in about 25%, and 5% is fully effective in an 18-hour test (Table II).

Sodium 2-methyl-1,4-naphthohydroquinone diphosphate was prepared by Fieser and Fry<sup>6</sup> and by Foster, *et al.*<sup>10</sup> The latter authors stated that it had an oral effectiveness lying below 2%. Our data (Table I) show that this substance has a potency of 1 unit in about 10%, and 5% is the minimum fully effective dose when the test period is prolonged to 18 hours (Table II). Compared with methylnaphthoquinone, this compound is but 1/20 as active. The results presented in Tables I and II do not agree with those of Foster, *et al.*,<sup>10</sup> who expressed the opinion that this sodium salt represents probably the most active antihemorrhagic substance known when compared on a molecular basis with methylnaphthoquinone. Furthermore, our observations do not support the speculation of these investigators as to whether the antihemorrhagic effects of 2-methyl-1,4-naphthoquinone, of its reduced form, and of like substances, are not mediated through a phosphoric ester.

We wish to report on still another water-soluble antihemorrhagic compound 2-methyl-1,4-naphthylene-dioxy diacetic acid. It has a potency of 1 unit in about 2 mg (Table I), and 100% is effective in

<sup>6</sup> Fernholz, E., and Ansbacher, S., *Science*, 1939, **90**, 315.

<sup>7</sup> Dann, F. P., *Proc. Soc. Exp. Biol. and Med.*, 1939, **42**, 663.

<sup>8</sup> Ansbacher, S., *J. Nutrition*, 1939, **17**, 303.

<sup>9</sup> Fieser, L. F., and Fry, E. M., *J. Am. Chem. Soc.*, 1940, **62**, 228.

<sup>10</sup> Foster, R. H. K., Lee, J., and Selmsen, U. V., *J. Am. Chem. Soc.*, 1940, **62**, 453.

sized by the isolation of such a material with a definite, though slightly diffuse boundary from crude vaccines less than 48 hours old (infectivity diminished at least 5 decimal dilutions) Older vaccines fractionated at pH below 7.0, however, yield materials that show only suggestions of a boundary or completely diffuse shadows in the sedimentation pattern where boundaries corresponding to this approximate particle size should appear

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### Water-Soluble Antihemorrhagic Compounds

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The interest in vitamin K analogues suitable for parenteral medication is evident from the current literature. Usually patients with hypoprothrombinemia due to obstructive jaundice receive orally vitamin K and bile or bile salts. Frequently these latter substances are badly tolerated and it seems that they are not needed, if the antihemorrhagic material is administered parenterally. In cases of severe hepatic insufficiency vitamin K orally is apparently not beneficial, but intravenous injections of a vitamin K analogue, such as e.g. 2-methyl-1,4-naphthoquinone, may be effective in raising the prothrombin content of the blood, as reported by Rhoads and Fliegelman.<sup>1</sup>

The first synthetic antihemorrhagic substance to be employed parenterally in the clinic was phthiocol.<sup>2,3</sup> Relatively large doses (50-100 mg) are required, because the potency of this compound is small.<sup>4</sup> Flynn and Warner<sup>5</sup> determined that phthiocol had but 1/500 the activity of methylnaphthoquinone, and we had reported pre-

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N. Y.

<sup>1</sup> Rhoads, J. E., and Fliegelman, M. T., *J. Am. Med. Assn.*, 1940, **114**, 400.

<sup>2</sup> Smith, H. P., Ziffren, S. E., Owen, C. A., and Hoffman, G. R., *J. Am. Med. Assn.*, 1939, **113**, 380.

<sup>3</sup> Butt, H. R., Snell, A. M., and Osterberg, A. E., *Proc. Staff Meeting Mayo Clin.*, 1939, **14**, 497.

<sup>4</sup> Ansbacher, S., and Fernholz, E., *J. Am. Chem. Soc.*, 1939, **61**, 1924.

<sup>5</sup> Flynn, J. E., and Warner, E. D., *Proc. Soc. Exp. Biol. and Med.*, 1940, **43**, 190.

to stand overnight. The product was then diluted with an equal volume of water and 10% hydrochloric acid added until an oil separated which soon solidified. It was removed by filtration, taken up in a 10% sodium carbonate solution, warmed in the presence of a decolorizing charcoal, filtered and reprecipitated with dilute hydrochloric acid. A pinkish powder with a m p of  $204-10^{\circ}$  was thus obtained. It was dissolved in a 10% sodium bicarbonate solution, treated three times with decolorizing charcoal and reprecipitated from the almost colorless solution with dilute hydrochloric acid. The final product was a white powder, m p  $217-218^{\circ}$ , yield 7.5 g (30%).

*Analysis* Calculated for  $C_{15}H_{14}O_6$ : C 62.04%, H 4.86%

Found: C 61.38% H 4.92%

Other water-soluble vitamin K substitutes have been prepared and studied clinically. According to Butt *et al.*<sup>11</sup> the 1,4-dihydroxy-2-methyl-3-naphthaldehyde of Binkley and coworkers<sup>12</sup> is effective at a level of 10 mg, and Broun<sup>13</sup> found that the 2-methyl-4-amino-1-naphthol hydrochloride of Doisy and associates<sup>14</sup> has a definite effect at 5 mg. Synthetic vitamin  $K_1$  is practically insoluble in water. It has been used by Frank *et al.*<sup>15</sup> in form of a colloidal suspension in glucose and was active at 10 mg. These data would seem to confirm the statement by Rhoads and Fliegelman<sup>1</sup> that "2-methyl-1,4-naphthoquinone appears to be the most potent agent for the treatment of prothrombin deficiency so far employed clinically."

*Summary* Methyl-naphthoquinone or methyl-naphthohydroquinone is the most active vitamin K compound known. It is as effective intravenously in an aqueous medium as orally in oil solution. *Per os* its potency is greater in water than in oil. The phosphate derivative is considerably less active. The sulfate is not as potent and not as rapidly absorbed by the organism as the phosphate. A new water-soluble vitamin K-active compound is described.

<sup>11</sup> Butt, H. R., Snell, A. M., Osterberg, A. E., and Bollman, J. L., *Proc. Staff Meeting Mayo Clin.*, 1940, **15**, 69.

<sup>12</sup> Binkley, S. B., Chenev, L. C., Holcomb, W. F., MacCorquodale, D. W., Thaver, S. A., and Doisy, E. A., 98th Meeting, Am. Chem. Soc. Boston, Sept. 12, 1939.

<sup>13</sup> Broun, G. O., 12th Ann. Meeting, Central Soc. Clin. Res., Chicago, Nov. 3 and 4, 1939 (*J. Am. Med. Assn.*, 1940, **114**, 440).

<sup>14</sup> Doisy, E. A., MacCorquodale, D. W., Thaver, S. A., Binkley, S. B., and McKee, R. W., Nation. Acad. Sci., Brown U. Meeting, Oct. 23-25, 1939 (*Science*, 1939, **90**, 407).

<sup>15</sup> Frank, H. A., Hurwitz, A., and Seligman, A. M., *New England J. Med.*, 1939, **221**, 975.

TABLE I  
Vitamin K Assays

Substance	Mode of administration	Vehicle	Response*		
			> 1 unit† γ	= 1 unit γ	< 1 unit γ
2 methyl 1,4 naphthoquinone‡	orally	oil	3/4	1/2	1/4
		water	1/2	1/4	1/10
	intraven	" §	3/4	1/2	1/4
Sodium 2 methyl 1,4 naphthohydroquinone diphosphate	orally	"	12.5	10	7.5
Sodium 2 methyl 1,4 naphthohydroquinone disulfate	"	"	30	25	20
Phthiocol	"	capsules	600	500	400
Purified phthiocol	"	"	2250	2000	1750
2 methyl 1,4 naphthylene dioxy diacetic acid	"	water	2250	2000	1750

\*A minimum of twenty severely vitamin K deficient chicks was used for each dose level

†Ansbacher unit (*J. Nutrition*, 1939, 17, 303)

‡Methylnaphthoquinone or methylnaphthohydroquinone

§Water or physiologic salt solution

TABLE II  
Minimum Effective Doses in 6 and 18 hour Tests

Substance	6 hr	18 hr	Ratio
	γ	γ	
2 methyl 1,4 naphthoquinone*	1/2† 1/4‡	1/4† 1/8‡	} 2:1
Sodium 2 methyl 1,4 naphthohydroquinone diphosphate	10	5	
Phthiocol (purified) 2 methyl 3 hydroxy 1,4 naphthoquinone	2000	1000	2:1
Sodium 2 methyl 1,4 naphthohydroquinone disulfate	25	5	5:1
Vitamin K <sub>1</sub> , 2 methyl 3 phenyl 1,4 naphthoquinone	15	1	15:1
2 methyl 1,4-naphthylene-dioxy diacetic acid	2000	100	20:1

\*Methylnaphthoquinone or methylnaphthohydroquinone

†Orally in oil or intravenously in an aqueous medium

‡Orally in water

an 18-hour test (Table II). This substance was prepared in the following manner:

To 15 g of 2-methyl-1,4-naphthohydroquinone and 16.3 g of monochloroacetic acid, 13.8 g of sodium hydroxide in 350 ml of water was added, the mixture refluxed for 6 hours under nitrogen and allowed

to stand overnight. The product was then diluted with an equal volume of water and 10% hydrochloric acid added until an oil separated which soon solidified. It was removed by filtration, taken up in a 10% sodium carbonate solution, warmed in the presence of a decolorizing charcoal, filtered and reprecipitated with dilute hydrochloric acid. A pinkish powder with a m p of  $204-10^{\circ}$  was thus obtained. It was dissolved in a 10% sodium bicarbonate solution, treated three times with decolorizing charcoal and reprecipitated from the almost colorless solution with dilute hydrochloric acid. The final product was a white powder m p  $217-218^{\circ}$  yield 7.5 g (30%).

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<sup>12</sup> Binklev, S. B., Chener, L. C., Holcomb, W. F., MacCorquodale, D. W., Thaver, S. A., and Doisy, E. A., 98th Meeting, Am. Chem. Soc., Boston, Sept. 12, 1939.

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<sup>15</sup> Frank, H. A., Hurwitz, A., and Seligman, A. M., *New England J. Med.*, 1939, 221, 975.



## Gonadotropic Response to Subcutaneous and Intraperitoneal Injections of Urine Prolan in White Rats

FELIX SULMAN AND JOACHIM SKLOW (Introduced by B. Zondek)

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Investigations by Collip<sup>1</sup> and Evans<sup>2</sup> showed that hypophyseal gonadotropic hormone is much more effective by subcutaneous than by intraperitoneal administration. With reference to gonadotropic hormone derived from pregnant mare serum (PMS), Evans<sup>2</sup> showed that it was just as effective intraperitoneally as subcutaneously. Later Pencharz<sup>3</sup> demonstrated that intraperitoneally PMS was 2 to 3 times more effective than subcutaneously, the degree of effectiveness, however depending upon the number of injections.

As to the effectiveness of intraperitoneal and subcutaneous administration of pregnancy urine prolان, Evans<sup>2</sup> reported "Pregnancy prolان, which contains no antagonist, is distinctly less effective intraperitoneally than subcutaneously. Here the difference seems to be due merely to faster absorption by the intraperitoneal route with consequent increased rate of excretion by the kidney or destruction by the tissues." The following experiments are intended to demonstrate that, provided a certain experimental procedure is used, it is possible to make pregnancy urine prolان develop its full effect also in the intraperitoneal route.

For the experiments, 72 infantile female rats, 3-4 weeks old and weighing 20-30 g were used. They received prolان by various methods, by a single injection, or 3 times at intervals of 24 hours or 6 times during 40 hours, either intraperitoneally or subcutaneously. Sixty hours after the first injection we began taking vaginal smears every 12 hours, and 120 hours after the first injection the animals were killed and their ovaries inspected.

Two prolان preparations were tested in this way: (1) "Purified prolان" (PP) which contained 333,333 RU per g; (2) Crude prolان (CP) which contained 1000 RU per g. The results of these experiments are shown in Table I.

As may be deduced from the experiments there is no fundamental

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<sup>1</sup> Collip, *Canad Med Assn J*, 1936, **34**, 458

<sup>2</sup> Evans, H. M., Korpi, K., Pencharz, R. I., and Simpson, M. E., *Univ Calif Publ Anat*, 1936, **1**, 237

<sup>3</sup> Pencharz, R. I., *Proc Soc Exp Biol and Med*, 1939, **42**, 525

difference between the effectiveness of purified prolan (PP) and crude prolan (CP) We shall only compare the results read from the estrous reaction (APR I)

One single injection of prolan was approximately 10 times (in case No 24 at least 6 times) as effective subcutaneously as intraperitoneally (Nos 2, 6, 20, 24) When, however, the injections were given in 3 portions the subcutaneous route was only twice as effective as the intraperitoneal one (Nos 9, 10, 26, 27) When the same amount of prolan was, however, distributed over 6 injections there was no longer any difference between intraperitoneal and subcutaneous administration (Nos 15 and 32)

Taking the corpus luteum reaction (APR III) as a standard almost the same values were obtained and it even seemed that with 6 intraperitoneal injections (*Cf* No 16) the APR III could be produced with smaller doses intraperitoneally than subcutaneously

Experiments Nos 15 and 32 represent the routine method of prolan standardization (Zondek<sup>4</sup>) in terms of estrous units using 6 injections If this experimental technic is adhered to it is therefore irrelevant whether the standardization is performed with subcutaneous or intraperitoneal injections

*Discussion* It was possible to verify the results obtained by Evans<sup>2</sup> that subcutaneous administration of pregnancy urine prolan proved to be more effective than intraperitoneal, provided only 1 to 3 injections were given When, however, the same amount of prolan was given in 6 portions, both methods were equally effective We are inclined to attribute these results to the more rapid absorption of the hormone from the intraperitoneal deposits, as explained by Evans<sup>2</sup> After only one to 3 injections it is very rapidly absorbed and its effectiveness considerably impaired Only when using 6 injections over 40 hours the absorptional conditions in the peritoneum become equal to those in the subcutis, so that optimal utilization of prolan is possible

When standardizing small amounts of prolan we had, moreover, the opportunity of studying the following phenomenon, which has been described for PMS by Hamburger<sup>5</sup> Distribution of 1 RU of prolan over several subcutaneous injections produces a less marked gonadotropic reaction than the administration of one single dose With larger doses, however, this does not hold true This phenomenon may be explained by the fact that, by splitting up a threshold dose

<sup>4</sup> Zondek, B, *Z Gebh and Gyn*, 1926, 90, 378, *Med Klin*, 1927, No 13, *Hormone d Ovariums u d Hypophysenvorderlappens*, Springer, Berlin, 1st ed, 1931, *cf* Zondek, B, and Aschheim, S, *Klin Wschr*, 1927, 6, No 6

<sup>5</sup> Hamburger, Ch, *Quart J Pharm and Pharmacol*, 1938, 11, 186

TABLE I

Exp No	Prolan	Injections	Doses Weight RU*	Gonadotropic reactions (APR) after subcutaneous injection			Gonadotropic reactions (APR) after intraperitoneal injection		
				I	II	III	I	II	III
				Estrous	Follicle hemorrhage	Corp lut	Estrous	Follicle hemorrhage	Corp lut
1	PP	1 x	1 gamma	-	-	-	-	-	-
2			15 "	+	-	-	-	-	-
3			3 "	+	-	-	-	-	-
4			6 "	+	-	-	-	-	-
5			9 "	+	-	-	-	-	-
6			15 "	+	-	-	+	-	-
7	PP	3 x	1 "	-	-	-	-	-	-
8			15 "	-	-	-	-	-	-
9			3 "	+	-	-	-	-	-
10			6 "	+	-	-	+	-	-
11			9 "	+	-	-	+	-	-
12			15 "	+	-	-	+	-	-
13	PP	6 x	1 "	-	-	-	-	-	+
14			15 "	-	-	-	-	-	-
15			3 "	+	-	-	+	-	-
16			6 "	+	-	-	+	-	-
17			9 "	+	-	-	+	-	-
18			15 "	+	-	-	+	-	-
19	CP	1 x	0.5 mg	-	-	-	-	-	+
20			1 "	+	-	-	-	-	+
21			15 "	+	-	-	-	-	+
22			3 "	+	-	-	-	-	+
23			45 "	+	-	-	-	-	+
24			6 "	+	-	-	-	-	+



(as 1 RU certainly is) several subthreshold doses are produced which cannot induce the gonadotropic response in the ovary

*Summary* Comparing the gonadotropic response of the organism to subcutaneous or intraperitoneal administration of pregnancy urine prolan the following results were secured (1) If the minimum dose is split up into 6 portions intraperitoneal and subcutaneous administration are equally effective (2) If the minimum dose is split up into 3 portions the intraperitoneal route is approximately half as effective as the subcutaneous one (3) If the minimum dose is given in one injection the intraperitoneal administration is about 1/10 as effective as subcutaneous (4) Using the subcutaneous standardization method with minute doses as *e g* 1 RU (estrous effect) the gonadotropic effect of 6 subthreshold doses is less marked than that of one single threshold dose

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### Cheilosis Successfully Treated with Synthetic Vitamin B<sub>6</sub>

SUSAN GOWER SMITH AND DAVID W MARTIN (Introduced by David T Smith)

*From the Department of Medicine and Department of Pediatrics, Duke University School of Medicine, Durham, N C*

Aykroyd and Krishnam<sup>1</sup> observed that the incidence of angular stomatitis (sores in the corners of the mouth perleche, cheilosis) coincided with a deficiency of some factor or factors of the vitamin B<sub>2</sub> complex

More recently Sebrell and Butler<sup>2</sup> produced these lesions in 10 out of 18 women subsisting on a riboflavin-deficient diet The lesions developed in a period of 94 to 130 days They failed to respond to nicotinic acid but responded to riboflavin the complete healing requiring from 5 to 58 days Sebrell and Butler<sup>3</sup> added more evidence to their previous findings and these have been confirmed by others<sup>4 5 6</sup>

<sup>1</sup> Aykroyd, W R, and Krishnam, B G, *Ind J Med Res* 1937 **24**, 707

<sup>2</sup> Sebrell, W H, and Butler, R E, *U S Pub Health Rep* 1938, **53**, 2882

<sup>3</sup> Sebrell, W H, and Butler, R E, *U S Pub Health Rep*, 1939, **54**, 2121

<sup>4</sup> Sjdenstricker, V P, Geeslin, L E, Templeton, C M, and Weaver, J W, *J A M A*, 1939, **113**, 1697

<sup>5</sup> Spies, T D, Vilter, R W, and Ashe, W F, *J A M A*, 1939, **113**, 931

<sup>6</sup> Jolliffe, N, Fein, H D, and Rosenblum, L D, *New Eng J Med* 1939

In view of these facts, it seems important to report our observations on 4 consecutive cases of cheilosis occurring spontaneously in association with other deficiency syndromes in patients admitted to this hospital and treated with synthetic vitamin B<sub>6</sub> [2 methyl, 3 hydroxy, 4 5 di(hydroxymethyl) pyridine] \*

The first patient treated was a 7-year-old colored girl admitted to the hospital with a diagnosis of pellagra. Pellagrous lesions were present on the hands and feet. A dietary history revealed a marked deficiency of the vitamin B-complex. There were typical cheilosis lesions of the lips characterized by maceration and fissuring at the angles (Fig 1a). There was an associated severe anemia. The patient was placed on the basic diet of Smith and Ruffin which is known to be low in the B-complex and especially so in riboflavin. Treatment with synthetic vitamin B<sub>6</sub> hydrochloride† was started with 20 mg intravenously 24 hours after admission. Within 5 hours slight but definite objective improvement could be observed in the mouth lesion. In 24 hours the improvement was demonstrable by

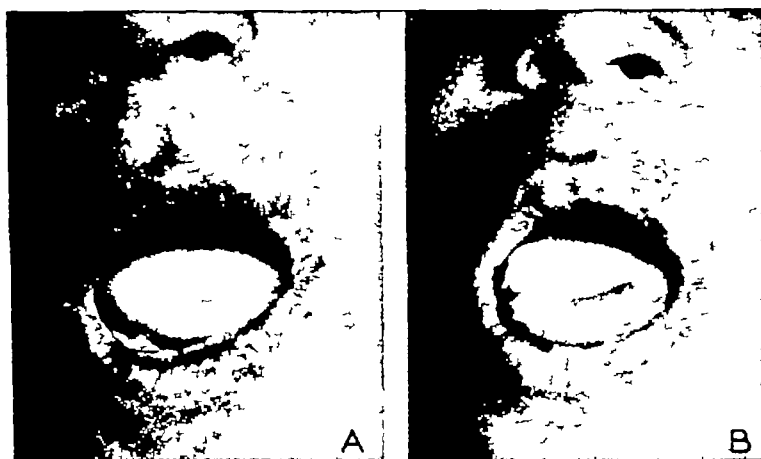


FIG 1

A Before treatment was initiated

B Five days after starting treatment with B<sub>6</sub>  
(20, 20, and 40 mg given 1st, 2nd, and 4th day respectively)

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\* The synthetic vitamin B<sub>6</sub> used in this work was obtained through the courtesy of Merck and Co, Rahway, N. J.

† Smith, D. T., and Ruffin, J. M., *Arch. Int. Med.*, 1937, 59, 631.

‡ The vitamin B<sub>6</sub> was prepared for intravenous use by dissolving it in freshly distilled water at a concentration of 50 mg per cc. The vitamin is readily soluble in water and no difficulty is encountered in getting this concentration. The solution was placed in a vaccine bottle and sterilized by autoclaving.

photograph A 20 mg dose was given intravenously the next day with continued improvement The next 2 days B<sub>6</sub> therapy was omitted and there was a slight regression At this time a 40 mg dose was given intravenously and the improvement obtained within 24 hours was marked (Fig 1b) The lesion healed completely from the effects of this dose This is the only anemia patient studied in which the anemia responded after B<sub>6</sub> treatment, while the patient was subsisting on a diet very low in iron

The second case of cheilosis occurred in a white woman, aged 62 years, suffering from severe gastric distress pain, nausea, distension There was an accompanying anorexia, anemia and extreme weakness of arms and legs The cheilosis was not quite so typical as in the first case because the fissures were not as prominent They were smaller, and tended to bleed and later form encrustations An inclination on the part of the patient to pick at these lesions resulted in some infection The first dose of 100 mg B<sub>6</sub> hydrochloride was given intravenously No treatment was given the next day On the third day 50 mg B<sub>6</sub> hydrochloride was given by mouth and a second 50 mg on the fourth day On the fifth day the lesions were entirely healed and did not return during the course of the illness though the patient died two months later with a blood dyscrasia The anorexia anemia and severe weakness were unaffected

The third case of cheilosis occurred in an 18-months-old white girl with a diagnosis of celiac disease Her lesions were characterized by small fissures in the corners of the mouth with red areas surrounding them They developed while she was subsisting on a diet composed primarily of milk and bananas At this time the only change of regime was the intravenous administration of 50 mg of B<sub>6</sub> hydrochloride daily for 4 days This resulted in the healing of the mouth lesions which remained healed during the rest of her stay in the hospital

A fourth case of cheilosis mild but characteristic occurred in a 27-year-old sprue patient This patient was extremely deficient in many of the vitamin factors There was slight improvement of the mouth lesion with B<sub>6</sub> but complete healing did not occur either with vitamin B<sub>6</sub> (1000 mg) or with a combination of B<sub>6</sub> (300 mg) riboflavin (140 mg) and nicotinic acid (1000 mg) The lesion finally healed completely however, when 445 units of a concentrated liver extract† was given intramuscularly over a period of one week

It is interesting in these 4 cases that the first was cured while receiving a basic diet deficient in riboflavin and the third developed

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† Lederle solution liver extract parenteral refined and concentrated (V.N.R.)

the lesion while receiving what should have been an adequate supply of riboflavin from approximately one quart of milk a day and a fair amount of vitamin B<sub>6</sub> in the banana which was assayed in this laboratory and found curative of the typical B<sub>6</sub> deficient dermatitis of rats when fed in daily doses of 0.75 g.<sup>8</sup>

We have found it true in controlled laboratory experiments that often a primary vitamin deficiency results in symptoms not altogether characteristic of that deficiency. This is brought about by the precipitation in the later stages of the primary deficiency of a secondary deficiency which is responsible for the symptoms observed.<sup>9</sup> It is probable that some such phenomenon may explain the curative powers of both riboflavin and B<sub>6</sub> in clinically similar lesions.

The nearest thing we have to an animal analogue of the cheilosis is the mouth lesion observed in the B<sub>6</sub>-deficient rat. This occurs when as much as 100 µg of synthetic riboflavin are fed individually but yields to treatment with synthetic B<sub>6</sub>. Similar lesions also occur in riboflavin-deficient rats but less frequently.

There are then 3 possible explanations of the observations that both riboflavin and B<sub>6</sub> cure cheilosis: (1) that riboflavin is the primary and specific deficiency responsible for the cheilosis and the B<sub>6</sub> operates only indirectly, (2) that vitamin B<sub>6</sub> is the primary and specific deficiency responsible for the cheilosis and the riboflavin acts only indirectly, and (3) that both riboflavin and vitamin B<sub>6</sub> are necessary to maintain the integrity of the lips at the mucocutaneous junction and that a deficiency of either will precipitate the lesion.<sup>10</sup>

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<sup>8</sup> Smith, S. G., unpublished data.

<sup>9</sup> Margolis, L. H., Margolis, G., and Smith, S. G., *J. Nut.* 1939, **17**, 63.

<sup>10</sup> Since this paper went to press two more patients with cheilosis have been treated, one with vitamin B<sub>6</sub> (350 mg) and one with riboflavin (50 mg) and both responded promptly. It should be noted here that Arkrovd and Krishnam<sup>10</sup> have reported the cure of cheilosis with a yeast preparation freed from flavin.

<sup>10</sup> Arkrovd, W. R., and Krishnam, B. G., *Ind. J. Med. Res.* 1938, **25**, 643.



Sulfathiazole Effect on *Staphylococcus aureus in vitro*

CHARLES H RAMMELKAMP AND CHESTER S KEEFER

*From the Thorndike Memorial Laboratory, Second and Fourth Medical Services (Harvard), Boston City Hospital, and the Department of Medicine, Harvard Medical School, Boston*

Recently several thiazole analogues of sulfapyridine have been described<sup>1</sup> and a few reports concerning their toxicity<sup>2</sup> and their protective action against *Staphylococcus aureus* in experimental animals<sup>3, 4</sup> have been made. It appears that the thiazole compounds may prove useful clinically. However, further information concerning their action both *in vitro* and in the experimental animal is desirable. It is for this reason that the present *in vitro* study with special emphasis on the action of sulfathiazole (2-sulfanilamidothiazole) on *S. aureus* was undertaken.

**Methods** Of the 4 strains of staphylococci used 3 were isolated from the blood stream of patients with bacteremia, and the other from a case of osteomyelitis of the femur. All 4 strains produced yellow pigment on agar, 2 produced hemolysis on blood agar, and all gave a strongly positive coagulase reaction.<sup>5</sup> The organisms were stored in the icebox on blood-agar slants, and 16-hour peptone-broth cultures were used. All dilutions were made in saline.

To study the comparative effects of sulfanilamide, sulfapyridine, sulfathiazole, and sulfamethylthiazole on staphylococci in whole blood the method employed previously by Spink and Keefer<sup>6</sup> was used. Defibrinated whole blood was obtained from individuals without signs of infection. To 0.5 cc of defibrinated blood 0.1 cc of various dilutions of the 16-hour broth culture was added. The chemicals were dissolved in saline and added in 0.1 cc amounts. The final concentration in each tube was 10 mg/100 cc unless otherwise stated. Tubes with 8 different dilutions of organisms were set up in duplicate, sealed in a gas-oxygen flame, and

<sup>1</sup> Fosbinder, R. F., and Walter, L. A., *J. Am. Chem. Soc.*, 1939, **61**, 2032.

<sup>2</sup> van Dyke, H. B., Greep, R. O., Rake, G., and McKee, C. M., *Proc. Soc. Exp. Biol. and Med.*, 1939, **42**, 410.

<sup>3</sup> Barlow, O. W., and Homburger, E., *Proc. Soc. Exp. Biol. and Med.*, 1939, **42**, 792.

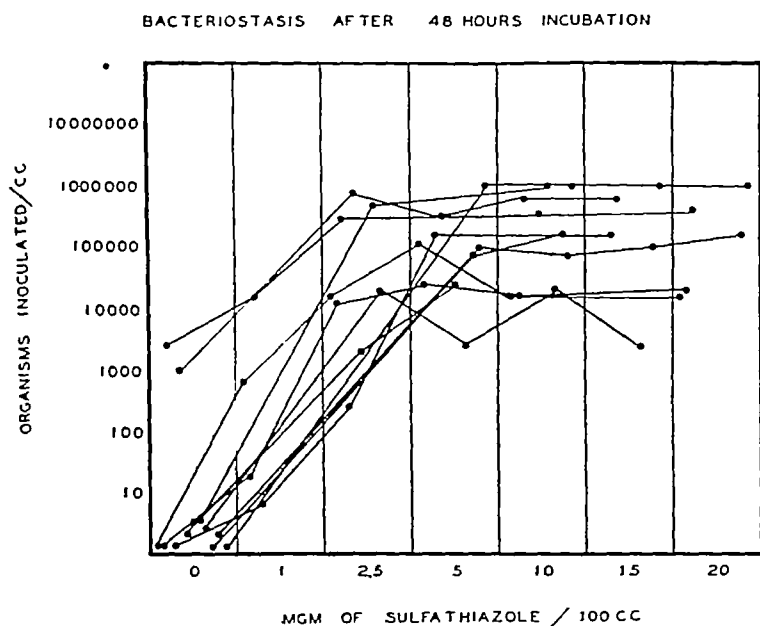
<sup>4</sup> McKee, C. M., Rake, G., Greep, R. O., and van Dyke, H. B., *Proc. Soc. Exp. Biol. and Med.*, 1939, **42**, 417.

<sup>5</sup> Chapman, G. H., Berens, C., Peters, A., and Curcio, L., *J. Bact.*, 1934, **28**, 343.

<sup>6</sup> Spink, W. W., and Keefer, C. S., *J. Clin. Invest.*, 1936, **15**, 21.

placed in a rotator in the incubator. The tubes were removed at 24 and 48 hours, and examined for hemolysis. Those tubes showing no hemolysis were then opened and the contents plated out and the colonies counted. The number of organisms present was calculated from the number contained in 2.5 cc of the sixth tube which was plated out at the beginning of the experiment.

*Action in whole defibrinated blood.* The majority of normal human defibrinated blood-samples could kill less than 10 staphylococci in 0.5 cc although rarely as many as 1000 organisms were killed. When sulfathiazole was added to these samples the killing power was increased, and there was evidence of definite bacteriostasis in the tubes in which there was growth. To determine the concentration that produced the maximal effect, dilutions of sulfathiazole were made so that final concentrations of 1, -2.5, -5, -10, -15, and 20 mg/100 cc were obtained. Chart I demonstrates the bacteriostatic action of sulfathiazole in these various concentrations. The evidence of bacteriostasis as represented here was the absence of hemolysis at the end of 48 hours. Hemolysis in these experiments did not occur unless there were  $10^8$  organisms per cc. In those tubes



Each dot represents the largest inoculum of *S. aureus* that failed to produce hemolysis after 48 hours' incubation. The lines connect individual experiments on the same sample of blood.

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<sup>6</sup> Spink, W. W., and Keefer, C. S., *J. Clin. Invest.*, 1936, **15**, 21.

addition of sulfathiazole to defibrinated whole blood which kills less than 10 staphylococci increased the bactericidal power at least 100 times. In 2 experiments in which the blood killed 50 to 100 organisms the addition of sulfathiazole enhanced the bactericidal effect 10 to 100 times. Again it is evident from this chart that concentrations of between 2.5 and 5 mg/100 cc gave effective results, and that increasing the level above this concentration did not enhance the bactericidal effect appreciably.

The above observations were repeated after giving sulfathiazole by mouth to normal persons. Blood was withdrawn both before and after the drug was administered, and the organisms were added. The second sample showed an increase in both the bactericidal and bacteriostatic action against the staphylococcus as compared with the sample of blood before the drug was given.

*Comparative effects of sulfanilamide derivatives.* Since sulfanilamide and sulfapyridine have both been disappointing in their effects

COMPARATIVE EFFECT OF VARIOUS SULFANILAMIDE DERIVATIVES  
ON PATHOGENIC STAPHYLOCOCCI

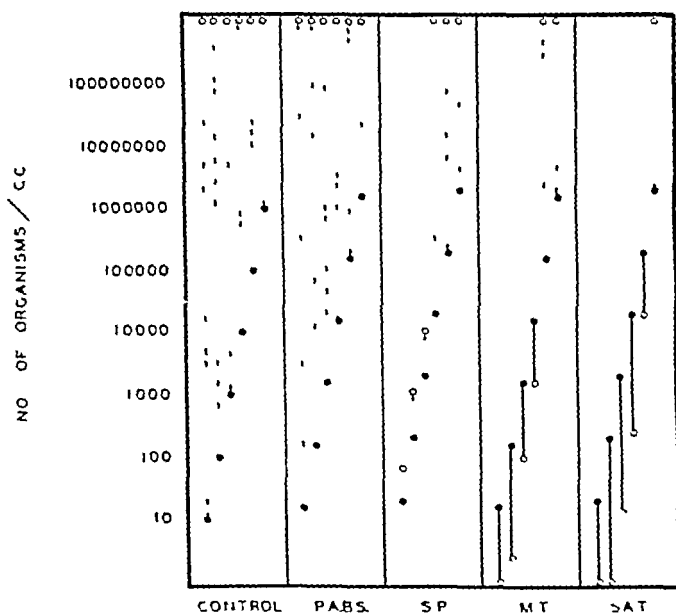


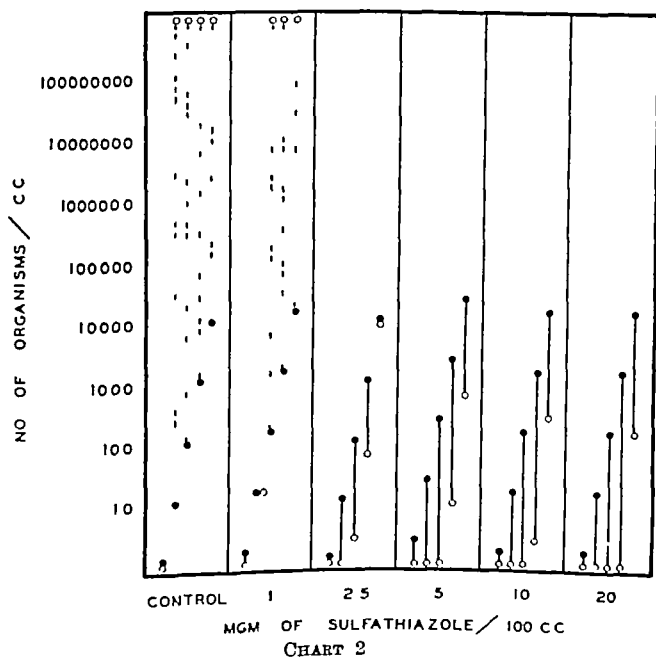
CHART 3

Solid dots represent original inoculum. Circles represent number of organisms per cc at the end of 48 hours' incubation. Broken lines indicate growth, solid lines indicate killing. P.A.B.S. = sulfanilamide, S.P. = sulfapyridine, M.T. = sulfamethylthiazole, S.A.T. = sulfathiazole.

showing no hemolysis there were  $10^4$  organisms or less per cc. Similar results were obtained when the tubes were examined at the end of 24 hours, but the color change was not so marked. In the tubes containing the drugs the hemolysis was greatly delayed as compared to the controls. From the chart it can be seen that the effective bacteriostatic level of sulfathiazole was between 2.5 and 5 mg/100 cc and that higher concentrations of the drug produced little increase in this action.

The increase in the bactericidal effect upon the addition of sulfathiazole was not as striking, but it was definite. Chart 2 represents a typical experiment on one sample of defibrinated blood. In all 12 such experiments were done. Again the results shown in this chart are taken after 48 hours' incubation in the rotator, however, the results at 24 hours were similar. Indeed there was little change in the number of organisms between the 24th and the 48th hour. The

BACTERICIDAL EFFECT OF VARIOUS CONCENTRATIONS OF SULFATHIAZOLE



Solid dots represent original inoculum per cc of *S aureus*. Circles represent final number of organisms per cc at the end of 48 hours' incubation. Solid lines indicate killing, broken lines growth.

## Effect of Insulin on Plasma Level and Excretion of Vitamin C\*

ELAINE P RALLI AND SOL SHERRY

*From the Department of Medicine, New York University College of Medicine*

In studying the metabolism of vitamin C in normal and depancreatized dogs, the urinary excretion of the vitamin was found to be strikingly reduced in the diabetic animal. The diets in both

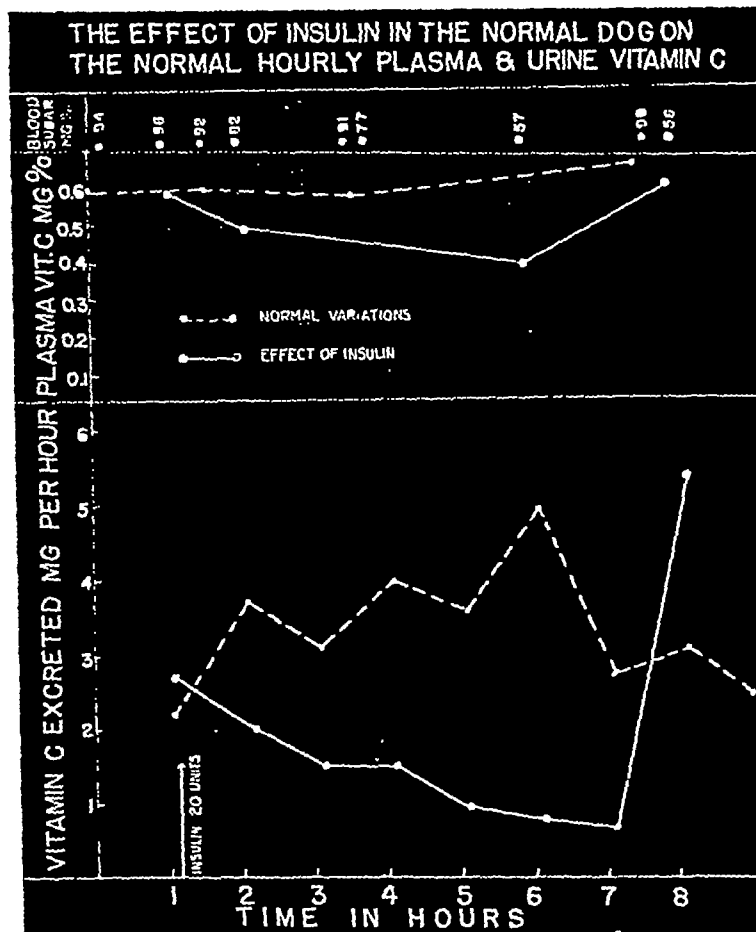


CHART 1

\* This research was aided by a grant from the Josiah Macy, Jr., Foundation

on the clinical course of cases with *S aureus* bacteremia, it seemed desirable to compare the action of these drugs with the thiazole compounds (sulfathiazole and sulfamethylthiazole) *in vitro*

The drugs were added to the blood so that the final concentrations were 10 mg/100 cc. In each of 8 experiments, the various drugs were added to the same sample of blood. 8 dilutions of organisms were made, and set up in duplicate tubes and incubated for 24 and 48 hours. The tubes were then opened and the contents cultured. Chart 3 illustrates the results of a typical experiment. When 10 or more organisms were added to the blood without any drug, and incubated for 48 hours, there were at least  $10^8$  organisms present at the end of 24 hours. With sulfanilamide the same was true. It was noted, however, that at the end of 24 hours there was evidence of bacteriostasis in that hemolysis of the blood was delayed. This, however, was never very striking. The bacteriostatic effect of sulfapyridine was shown when small numbers of organisms were used, that is less than 10,000 per cc of blood. Both sulfathiazole and sulfamethylthiazole showed bacteriostatic and bactericidal properties. The numbers of organisms actually decreased during incubation and this was striking even when as many as 100,000 organisms per cc were used. From our experiments, using whole blood, sulfathiazole was slightly superior to sulfamethylthiazole although the difference was not great. It seems justifiable to say that in whole defibrinated blood, sulfathiazole is more effective against staphylococci than sulfamethylthiazole, sulfapyridine, or sulfanilamide.

**Conclusions** Sulfathiazole is an effective bacteriostatic and bactericidal agent against *S aureus* when added to defibrinated whole blood *in vitro*. Concentrations between 2.5 and 5 mg/100 cc are necessary to obtain the maximal effect. Comparison of this drug with sulfamethylthiazole, sulfanilamide, and sulfapyridine show it to be somewhat superior in the experiments reported.

We wish to thank The Squibb Institute of Medical Research for the supply of sulfathiazole and The Department of Medical Research of the Winthrop Chemical Company for the supply of sulfamethylthiazole used in this investigation.

We wish to thank Miss Marjorie Jewell and Miss Eleanor Fleming for technical assistance.

# THE EFFECT OF INSULIN IN THE DIABETIC DOG ON THE NORMAL HOURLY PLASMA & URINE VITAMIN C

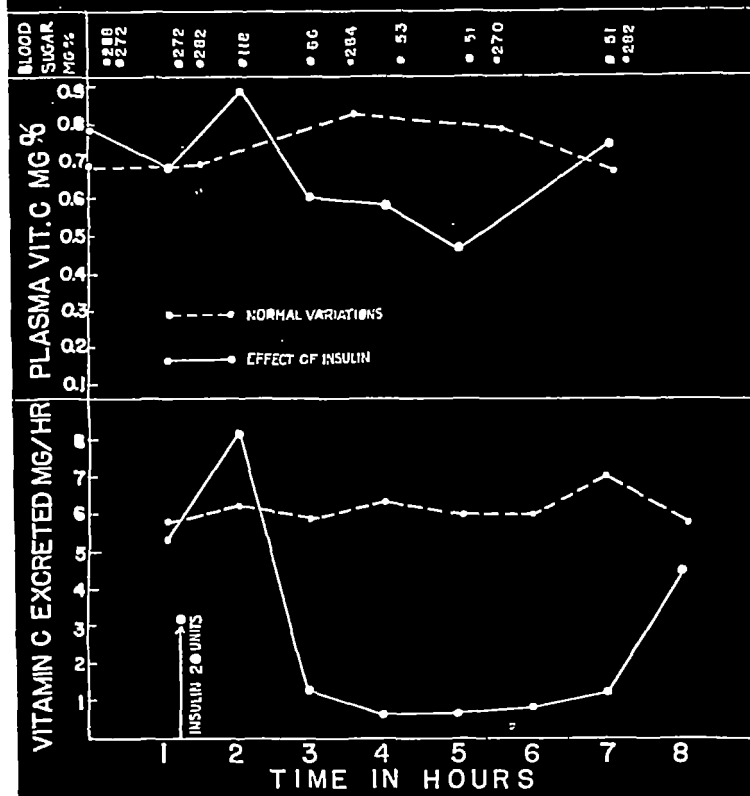


CHART 2

the urinary excretion varied from 2.16 to 4.92 mg per hour (Chart 1). In the diabetic dog the plasma levels of vitamin C varied from 0.69 to 0.83 mg % and the urinary excretion from 5.70 to 7.04 mg per hour (Chart 2). In 3 experiments in the normal dog 20 units of insulin were injected subcutaneously and at the same time a small dose of glucose was given by mouth to avoid too rapid a fall in the blood sugar. One typical experiment is shown in Chart 1. In contrast to the normal variations in plasma levels of vitamin C, following the injection of insulin there is a fall in the plasma concentration and the urinary excretion of the vitamin. The effect lasted about 6-7 hours at which time both the plasma level and the urinary excretion returned to normal.

In the diabetic dog (Chart 2) in 3 experiments the results are



groups of animals were totally devoid of vitamin C dried meat powder was used in place of raw beef. The dogs were kept in individual metabolism cages and the urines were collected by catheter at the start and end of each 24-hour period. Urines voided between catheterizations were collected in dark bottles containing sulphuric acid and hydroxyquinoline to prevent the destruction of the urinary ascorbic acid. Fasting bloods were taken for the determination of the plasma vitamin C.<sup>1</sup> Urinary vitamin C was determined by the method of Evelyn.<sup>2</sup> The depancreatized dogs received insulin twice daily in amounts which allowed a moderate daily excretion of sugar.

The 6 normal dogs excreted an average of 20.6 mg of vitamin C per kg of body weight in 24 hours. The fasting plasma levels of vitamin C varied from 0.55 to 0.94 mg %. The 7 diabetic dogs maintained on insulin excreted on an average only 3.6 mg of vitamin C per kg of body weight in 24 hours. The fasting plasma levels varied from 0.33 to 0.71 mg %.

The influence of injected insulin on the plasma level and excretion of vitamin C was studied in a series of acute experiments in both the normal and depancreatized dog. Control experiments were run on each dog to ascertain the hourly plasma variation and urinary excretion of vitamin C. Over a period of 8 hours the plasma levels of vitamin C in the normal dog varied from 0.58 to 0.66 mg % and

TABLE I.

Average 24 hour Excretion of Vitamin C and Fasting Plasma Levels in Normal and Depancreatized Dogs on Insulin

Dog No	Wt, kg	No daily observations	Urinary excretion vitamin C in mg daily		Urinary excretion per kg Body wt	Fasting plasma levels vitamin C mg %			
			Avg	S.D.		No	obs	Avg	S.D.
207	15.75	13	304	±32.1	19.3	5		0.55	±0.09
211	12.25	17	189	±20.6	15.4	8		0.65	±0.07
197	11.25	10	232	±17.0	20.6	7		0.62	±0.14
193	9.0	21	163	±18.3	18.1	13		0.63	±0.08
215	11.75	1	300		25.5	1		0.89	
218	13.0	6	319		24.5	2		0.94	
Diabetic Dogs *									
192	9.75	25	14	±6.5	1.4	9		0.45	±0.03
214	6.75	5	8	±1.6	1.2	2		0.41	
216	8.25	5	16	±2.0	1.9	2		0.33	
170	12.50	15	71	±21.0	5.9	5		0.49	±0.12
211	10.50	13	81	±9.0	7.7	6		0.71	±0.05
215	10.50	11	29	±11.0	2.8	5		0.66	±0.12
208	11.50	5	52	±13.0	4.5	2		0.66	

\*The depancreatized dogs received insulin twice daily

1 Mindhn, R. L., and Butler, A. M., *J Biol Chem*, 1938, **122**, 673

2 Evelyn, K. A., Malloy, H. T., and Rosen, C., *J Biol Chem* 1938, **120**, 655

TABLE I  
Growth from Contaminated Serum (1.0 and 0.25 cc Amounts)

Preserved with	In thioglycollate medium Contaminant		In infusion broth (Smith tubes) Contaminant	
	<i>Staph aureus</i>	<i>Cl tetani</i>	<i>Staph aureus</i>	<i>Cl tetani</i>
Merthiolate, 1:5000	+	+	—	—
" 1:10,000	—	+	—	—
Phenol, 0.5%	+	+	—	—
No preservative	+	+	+	—

were available dried spores of *Cl tetani* in sand, 10 years old prepared by Coleman.<sup>4</sup> Vaccines and serums were inoculated lightly with these organisms singly and in mixtures. From these products 0.25 cc and 1.0 cc respectively containing from 5 to 500 organisms, were transferred to standard Smith tubes and also to tubes containing 12 cc of thioglycollate medium.\* Incubation at 37°C for 7 days followed with frequent observations.

A summary of results is given in Table I. No difference between results with vaccines and serums was noted. A hundred-fold increase in the inocula did not change the results. *Ps. pyocyanea* appeared to be less sensitive to merthiolate than other organisms, serum preserved with merthiolate, 1:10,000, usually yielded growth and, after several days, serum with merthiolate, 1:5000 usually yielded growth, even in ordinary broth. Dried spores of the *Cl tetani* failed to develop in Smith tubes in pure culture, although they grew quickly in thioglycollate medium. They failed to develop in Smith tubes in pure culture even from unpreserved serum or serum preserved with phenol. Numerically, an inoculum of 150 spores, the maximum tested failed to grow in Smith tubes. 10 spores the lowest number tested grew readily in thioglycollate medium. In fact, growth of these spores in Smith tubes was observed only when aerobes were also present and this was irregular.

In summary it seems clearly evident that the standard test for sterility is inadequate because of the inhibition of growth of possible contaminants by merthiolate or similar bacteriostatic substances and because the method is not reliable for anaerobic contaminants. It seems equally clear that thioglycollate medium is adapted to meet these objections and with suitable refinements should replace the standard test for sterility.

<sup>3</sup> Rosenstern, C., Levin, I. and Levin, H., *Am J Hyg.*, 1935, **21**, 260.

<sup>4</sup> Coleman, G. E., *J Infect Dis* 1930 **47**: 410.

\* Furnished by the Baltimore Biological Laboratories. The formula is: pork infusion from 37.5 g per liter, thiopeptone, 1% dextrose, 1% NaCl, 0.5% Na thioglycollate, 0.1% agar, 0.05% methylene blue, 0.0002%.

similar There was a sharp fall in the plasma level and urinary excretion of vitamin C as soon as the subcutaneously injected insulin became effective

In additional experiments we have found that if glucose is administered intravenously at the time that the plasma vitamin C level and urinary excretion is reduced the effect can be overcome and the plasma level of the vitamin returned to normal

Similar studies are being made at present on human normal and diabetic subjects

## 11298 P

### Test for the Sterility of Biologic Products

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Standard tests for the sterility of finished vaccines or serums consist of the inoculation of 0.25 cc and 1.0 cc from the proper number of samples of the product to Smith fermentation-tubes containing infusion-broth. The tubes are heated within 5 hours before inoculation to drive off dissolved oxygen. They are then inoculated and are incubated for 7 days. The presence of weak points in the standard procedure has long been suspected.

The inherent weaknesses of the standard test are, in part, dependent upon (1) the frequent use of merthiolate which, because of its bacteriostatic effect, prevents the growth of contaminants, if any, in many instances, and (2) standard Smith tubes are not well adapted to the cultivation of anaerobes. The use of a second transfer, after 7 days, from optically clear Smith tubes to fresh Smith tubes in an endeavor to overcome the bacteriostatic action of the preservative is definitely irrational and thus unsatisfactory.<sup>1</sup>

The use of Brewer's medium<sup>2</sup> containing thioglycollate overcomes the objections given because it permits the growth of organisms in the presence of merthiolate and because it is suited to the cultivation of anaerobes. The results reported here support these views.

Tests were made with vaccines (bacterins) and serums, preserved with merthiolate, 1:5000 or 1:10,000 or with phenol, 0.5%. As contaminants 24-hour cultures of *Staph aureus*, *Staph albus*, *Ps pyocyanea*, *B subtilis* and *C verosis* were used.<sup>3</sup> In addition, there

<sup>1</sup> Marshall, M. S., and Hrenoff, A. K., *J Infect Dis*, 1937, **61**, 42

<sup>2</sup> Brewer, J. H., *J Bact*, 1940, **30**, 10

estrus of the rats. The "phaseolin", however, as prepared by Zselyonka and Illenyi,<sup>2</sup> inhibited the estrus when added at the same level to the basic food. Osborne's method differs substantially from the other in that it involves a thorough extraction of the bean proteins with lipid solvents.

3. The raw bean flour mixed in the diet to 15% inhibited the estrus of rats. However, the same bean flour was found ineffective in this sense even at a 20% level, after its thorough extraction in a Soxhlet apparatus or after repeated thorough extraction at room temperature with fat solvents.

4. Furthermore, a crude oil extracted from the bean flour with fat solvents at room temperature in about 17% yield inhibited the estrus of the rats during the period of its subcutaneous administration. Controls receiving the same amount of olive oil continued to show their regular estrus.

5. It was found that this estrus-inhibiting principle in the oil of the white bean, as extracted with lipid solvents, is directly antagonistic in its action on castrated female rats to the estrogenic hormone. One gamma estrogenic hormone (international standard ketohydroxyestrin) injected subcutaneously first proved to be sufficient in our castrated rats to evoke estrus in about 100%. In cross-experiments the same amount of estrogen was found to be 50% less effective when the test groups were kept on a 15% by weight bean diet prior to, during, and after the administration of the estrogenic hormone.

The results of these experiments seemed to indicate that in the lipid-soluble fraction of the bean there is present a factor with a biological action similar to that of progesterone.

6. In order to examine this point further, the crude bean oil was tested in the Clauberg test, known as a most specific one for progesterone-like effects. We used the abbreviated form of the Clauberg test as proposed by Butenandt *et al.*<sup>3</sup>

With this test a positive progesterone-like effect of the bean oil could be repeatedly demonstrated. This is the first example of a substance from a plant source which exerts a progesterone-like effect.

This progesterone-like principle of the bean was found to be very sensitive to heat, oxidative agents, and alkalies. Only extracts which took account of these properties retained their potency.

<sup>2</sup> Osborne, T. B., and S. Raue, E., *Abderhalden's Handbuch d. biol. Arbeitsmethoden*, Abt. I, Bd. 8, p. 425.

<sup>3</sup> Butenandt, A., Westphal, U., and Holweg, W., *Z. physiol. Chem.* 1934, **227**, 84.

## Presence of a Principle with Progesterone-like Activity Obtained from a Plant Source

G DE SUTO-NAGY \* (Introduced by C N H Long )

*From the Institute of General Pathology, University of Budapest*

The known biological incompleteness of the protein fraction of the bean has received considerable attention in interpretations of the malnutrition caused by a raw bean diet. Belák and Szathmáry,<sup>1 2</sup> and Zselyonka and Illényi<sup>3</sup> were the first to point out that together with this loss of weight there was also observable an inhibition of the estrus of mice during the period of feeding with a diet containing 15% of raw bean. They isolated a globulin fraction from the bean flour, which they referred to as "phaseolin". Together with a loss of weight, the inhibition of the estrus of the mice could be observed when 5% of the basic food of the mice was replaced by this protein fraction. The fat-soluble fraction of the bean was found by the same authors to be ineffective. Selye and Collip,<sup>4</sup> in replacing 30 to 50% of the basic food of the rat with bean flour, observed a certain atrophy of the ovaries and interpreted their findings as a consequence of a "malnutrition".

In our feeding experiments, first using rats as test animals, and following the course of the estrous cycles by means of the vaginal smear method of Long and Evans,<sup>5</sup> we were able to establish the following

1 Complete inhibition of the normal estrous cycle could be observed when 15% of the basic food was replaced by bean flour. Controls, on a diet without bean flour, failed to show any change in their regular cycles whether that diet was offered to them (a) *ad libitum*, or (b) in an amount 15% less by weight than that regularly taken by these animals.

2 The pure globulin fraction of the bean as prepared by Osborne,<sup>6</sup> given in 5% by weight to the basic food did not inhibit the

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\* Present address, Department of Chemistry, Yale University, New Haven, Conn.

<sup>1</sup> Belák, S, and Szathmáry, J, 5th Congress of the Hungarian Physiol Soc, Debrecen, June, 1935

<sup>2</sup> Belák, S, and Szathmáry, J, *Biochem Z*, 1937, **291**, 259

<sup>3</sup> Zselyonka, L, and Illényi, A, *Biochem Z*, 1937, **291**, 263

<sup>4</sup> Selye, H, and Collip, J B, *Endocrinology*, 1936, **20**, 667

<sup>5</sup> Long, J A, and Evans, H., *Memoirs of the Univ of California*, 1922, **6**

purified thrombin and the amount that disappeared from solution calculated. The conversion of 1 mg of fibrinogen resulted in the adsorption of 51 units of thrombin upon the fibrin strands, no further adsorption occurred regardless of the time of incubation in the presence of excess amounts of thrombin. Therefore when blood coagulates, approximately 90% of the thrombin must be inactivated or neutralized by the normal antithrombic activity of the serum.

Observations were then made on the quantitative activity of the antithrombin of diluted and undiluted serum and oxalated plasma. The fibrinogen was removed from plasma by incubation for 10 minutes at 56°C. When 1 unit of thrombin was added to 1 cc of various dilutions of serum and plasma and the amount of thrombin calculated that was inactivated at various periods of incubation at 28°C, it was observed that there was a quantitative correlation in antithrombic activity at 4 minutes' incubation in dilutions greater than one part in forty-five. There was little or no difference in the antithrombin of serum and plasma. In the ultimate test the incubated (56°C, 10 minutes) serum or plasma was diluted (usual dilutions, 1 part in 40, 50, or 60) to such an extent that 1 cc would inactivate 0.48 to 0.59 units of thrombin in 4 minutes at 28°C. In such dilutions no correction was necessary for the thrombin-neutralizing activity of oxalates. However, when the results of such a test were compared with the ability of undiluted serum or plasma to neutralize or inactivate thrombin the calculated result had to be multiplied by a correction factor of 5.28. The addition of small amounts of thrombin to undiluted serum or plasma was no index of the antithrombic activity because of the unexplained phenomenon that small fractions of a unit of thrombin may remain active for considerable time. One unit of antithrombin is defined as that amount which will neutralize or inactivate 1 unit of thrombin in 4 minutes incubated at 28°C.

TABLE I  
Normal Levels of Antithrombin in Human Subjects and Various Animals

Source of blood	No. of subjects	Avg antithrombin units per cc	Range of variation units per cc*
Human, normal	34	90	74-115
Dog	15	87	74-105
Cat	5	96	79-117
Cow	11	95	82-119
Pig	4	103	101-109
Guinea pig	10	108	97-120
Rabbit	18	108	98-133
Rat (albino)	28	123	102-147

\*Lowest and highest determinations

## Quantitative Studies on Antithrombin \*

SLOAN J WILSON (Introduced by E von Haam )

*From the Department of Pathology, Ohio State University, Columbus, Ohio*

The normally existing anticoagulants of blood serum and plasma according to present concepts are antiprothrombin and antithrombin. Howell<sup>1</sup> stated that blood normally contains but a small amount of antithrombin. A critical analysis of previous methods for the quantitative determination of antithrombin has only become possible with the purification and standardization of prothrombin and thrombin by Warner, Brinkhous, Smith and Seegers.<sup>2</sup> Previous methods have consisted of either adding considerably less than one unit of thrombin to undiluted serum and observing the clotting time upon the subsequent addition of fibrinogen after various periods of incubation<sup>3, 4, 5</sup> or by adding a small fraction or a few units of thrombin to plasma and observing the variations in the clotting time.<sup>6, 7</sup> The obvious conclusion was that serum or plasma can inactivate or neutralize very little thrombin. It is well established that hemorrhage may occur with plasma prothrombin levels of 35% of normal (105 units) or less. Theoretically there should be a marked excess of prothrombin even in this hemorrhagic zone inasmuch as one unit of prothrombin when converted to thrombin will convert 1 cc of fibrinogen to fibrin in 15 seconds. From these observations it would seem probable that the antithrombic activity of serum and plasma is much greater than previously assumed.

Before a method was devised for the quantitation of antithrombic activity of serum or plasma, the quantitative adsorption of thrombin was observed during the conversion of fibrinogen to fibrin. To various amounts of purified fibrinogen were added 60 units of

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\* Aided by a grant from the Comly Research Fund, Ohio State University

1 Howell, W H, *Physiol Rev*, 1935, **15**, 435

2 Warner, E D, Brinkhous, K M, and Smith, H P, *Am J Physiol*, 1936, **114**, 667, Smith, H P, Warner, E D, and Brinkhous, K M, *J Exp Med* 1937, **66**, 801, Seegers, W H, Brinkhous, K M, Smith, H P, and Warner, E D, *J Biol Chem*, 1938, **126**, 91

3 Howell, W H, *Arch Int Med*, 1914, **13**, 76

4 Gasser, H S, *Am J Physiol*, 1916, **42**, 378

5 Mills, C A, and Kitzmiller, K V, *Arch Int Med* 1926, **38**, 544 *ibid*, 1927, **39**, 618

6 Quick, A J, *Am J Physiol*, 1938, **123**, 712

7 Eagle, H, *Bull Johns Hopkins Hosp*, 1937, **60**, 428

purified thrombin and the amount that disappeared from solution calculated. The conversion of 1 mg of fibrinogen resulted in the adsorption of 5.1 units of thrombin upon the fibrin strands, no further adsorption occurred regardless of the time of incubation in the presence of excess amounts of thrombin. Therefore when blood coagulates, approximately 90% of the thrombin must be inactivated or neutralized by the normal antithrombic activity of the serum.

Observations were then made on the quantitative activity of the antithrombin of diluted and undiluted serum and oxalated plasma. The fibrinogen was removed from plasma by incubation for 10 minutes at 56°C. When 1 unit of thrombin was added to 1 cc of various dilutions of serum and plasma and the amount of thrombin calculated that was inactivated at various periods of incubation at 28°C, it was observed that there was a quantitative correlation in antithrombic activity at 4 minutes' incubation in dilutions greater than one part in forty-five. There was little or no difference in the antithrombin of serum and plasma. In the ultimate test the incubated (56°C, 10 minutes) serum or plasma was diluted (usual dilutions 1 part in 40, 50, or 60) to such an extent that 1 cc would inactivate 0.48 to 0.59 units of thrombin in 4 minutes at 28°C. In such dilutions no correction was necessary for the thrombin-neutralizing activity of oxalates. However, when the results of such a test were compared with the ability of undiluted serum or plasma to neutralize or inactivate thrombin the calculated result had to be multiplied by a correction factor of 5.28. The addition of small amounts of thrombin to undiluted serum or plasma was no index of the antithrombic activity because of the unexplained phenomenon that small fractions of a unit of thrombin may remain active for considerable time. One unit of antithrombin is defined as that amount which will neutralize or inactivate 1 unit of thrombin in 4 minutes incubated at 28°C.

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\*Lowest and highest determinations



The antithrombin of the serum and plasma of various species was then determined (Table I). Human serum and plasma contain between 74 and 115 units per cubic centimeter. In human subjects with the plasma prothrombin within the hemorrhagic zone the prothrombic unitage usually approximated or was lower than the antithrombic unitage.

*Summary* A method is devised for the quantitative determination of antithrombic activity of serum and plasma. One unit of antithrombin is defined as that amount which will inactivate or neutralize 1 unit of thrombin in 4 minutes' incubation at 28°C. There is little or no quantitative difference in the antithrombin of serum and plasma. The antithrombic activity of normal serum and plasma is considerably greater than has been previously described.

## 11301 P

### Diphtheria-Antitoxin Production After Intravenous or Subcutaneous Injection of Alum-Toxoid

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The purpose of the present study is to compare the effectiveness of intravenous and subcutaneous injections of alum-precipitated diphtheric toxoid in stimulating antitoxin-formation in the rabbit. The general opinion that the subcutaneous is superior to the intravenous route in antitoxin-production is based on experiments with horses<sup>1</sup> and guinea pigs.<sup>2</sup> Further important difference between the previous and the present experiments is that in the previous studies the antigen, toxin or toxoid, was used in solution and in the present study the toxoid was employed as particulate material.

When bacterial suspensions are employed as antigen the vascular route is the more effective one. This is true not only in regard to pneumococci that rapidly become gram-negative when introduced into the skin,<sup>3</sup> but also in regard to heat-killed tubercle bacilli that

<sup>1</sup> Kolle, W., and Wassermann, A., *Handbuch der path. Mikroorganismen*, 1912, Verlag Gustav Fischer, Jena.

<sup>2</sup> Neill, J. M., Sugg, J. Y., and Richardson, L. V., *J. Immunol.*, 1935, **28**, 363.

<sup>3</sup> Julianelle, L. A., *J. Exp. Med.*, 1930, **51**, 441, 449, Dubos, R. J. and MacLeod, C. M., *J. Exp. Med.*, 1938, **67**, 269.

are quite resistant<sup>4</sup> The question can be raised whether toxins or toxoids would behave like bacteria if they are injected as particulate material By the subcutaneous route toxins and toxoids in combination with particulate material or as precipitates were employed by Ramon<sup>5</sup> and Glenn<sup>6</sup> and his associates<sup>6</sup>

Four rabbits received intravenous and 5 rabbits subcutaneous injections of 1 cc of alum-precipitated diphtheric toxoid containing 25 L<sub>t</sub> on 4 successive days and after a rest of 3 days a similar course of 4 injections The antigen was diluted 1:10 with salt solution for the intravenous injections The animals were tested 12, 18, and 25 days after the first course of 8 injections Thirty-two days after the first course of injections the rabbits were given a new series of injections, but this time 5 days apart The rabbits were bled approximately 5 days after each injection The titrations were carried out by the intracutaneous method

The results are presented in Table I The first 3 samples of blood taken at different intervals after the first series of 8 injections show that the rate of antitoxin-formation was strikingly higher in the group with intravenous injections, since at the time of the first bleeding the average level in this group was 1.8 and in the group with subcutaneous injections only 0.1 antitoxin unit per cc At the

TABLE I  
Antitoxin Units per cc of Serum in Rabbits Immunized with Alum precipitated Diphtheric Toxoid.

Route	Rabbit No	Days after the first course of injections			During the second course of injections						
		12	18	25	After						
Intravenous	1	5.0	4.0	2.0	15.0	15.0	20.0	15.0	12.5	12.5	10.0
	2	1.0	1.0	2.0	11.0	15.0	20.0	—	18.0	18.0	15.0
	3	1.0	2.0	1.0	11.0	12.0	15.0	15.0	10.0	10.0	7.5
	4	1.0	1.0	0.5	3.0	7.0	7.5	—	7.5	7.5	5.0
Avg		1.8	2.0	1.3	10.0	12.2	15.6	*16.0	12.0	12.0	9.3
Subcutaneous	5	1	2.0	5	3.0	7.0	5.0	5.0	3.0	2.5	2.5
	6	1	1.0	5	3.0	6.0	5.0	2.5	2.0	3.0	2.5
	7	1	2.0	5	3.0	5.0	4.0	2.5	3.0	2.5	2.5
	8	1	1.0	5	2.0	5.0	4.0	2.5	3.0	2.5	2.5
	9	1	0.1	1	1.0	3.0	3.0	2.5	2.0	3.0	2.5
Avg		1	1.2	4	2.4	5.2	4.2	2.5	2.7	2.8	2.5

\*Pooled sera

<sup>4</sup> Freund, J., and Opie, E. L., *J. Exp. Med.*, 1938, **68**, 273

<sup>5</sup> Ramon, G., *Revue d'Immunol.*, 1939, No. 5, 385

<sup>6</sup> Glenn, A. T., Pope, C. G., Waddington, H., and Wallace, U., *J. Path. and Bact.*, 1926, **20**, 38

time of the second and third bleedings the level was only slightly higher in the animals with intravenous injections. The 2 injections of the antigen that followed the rest-period stimulated a sharp rise in the titers in both groups while the following injections produced a rise only in the group with intravenous injections. At the time of all of the 7 bleedings during the second course of injections the antitoxin levels were conspicuously higher in the group that received intravenous injections.

*Conclusion* Antitoxin-formation in the rabbit is more rapid and abundant after intravenous than after subcutaneous injections of 25 L<sub>1</sub> alum-precipitated diphtheric toxoid.

## 11302

### Experiments on Cultivation of Virus of Infectious Avian Encephalomyelitis \*

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*From the Laboratories of the Rockefeller Institute for Medical Research, New York*

In connection with investigations on the virus of infectious avian encephalomyelitis (A. E.),<sup>1</sup> we sought to enhance antigen quantitatively for the preparation of immunizing vaccines, by cultivating the virus in developing chick embryos and in their tissues *in vitro*. The results relating to epidemiology and to additional properties of this newly discovered virus were considered of sufficient importance to be offered in the present paper.

*Materials and Methods* The strain of virus employed was that kindly sent to us by Dr. Van Roekel and which was described by him<sup>1</sup> as well as by Olitsky.<sup>1</sup> The procedures followed closely those which had been employed by the latter. Ten percent infected-chick-brain suspension in broth was used to initiate the various cultures. Tests for virus, except as noted otherwise, were made by intracerebral inoculation of 0.05 to 0.1 cc of tenfold dilutions of the material to be examined into 2- or 3-weeks-old Rhode Island, or New Hampshire, Red chicks.

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\* We express our debt to Mr. P. Haselbauer for his invaluable aid.

<sup>1</sup> Olitsky, P. K., *J. Exp. Med.*, 1939, **70**, 565; Olitsky, P. K., and Bauer, J. H., *Proc. Soc. Exp. Biol. and Med.*, 1939, **42**, 634. For earlier descriptions, see Jones, E. E., *J. Exp. Med.*, 1934, **59**, 781, and Van Roekel, H., Bullis, K. L., and Clarke, M. K., *J. Am. Vet. Med. Assn.*, 1938, **NS 46**, 372.

Fertile eggs of White Leghorn chickens were available. It was shown that of 11 normal, 2-week-old birds of this breed given 0.05 cc of  $10^{-1}$  dilution of active brain intracerebrally, all developed characteristic encephalomyelitis after 11 to 16 days. Hence the Leghorn chicks are susceptible to the A. E. virus.

*Cultivation Experiments* 1. *In Developing Chick Embryos* In view of the fact that the virus is active only in avian hosts and that it has been reported<sup>2</sup> that the disease can be transmitted by way of the egg, the first attempts at cultivation were made in embryonated eggs as the most promising approach to the problem. Since the virus was shown to be active in nervous tissue, it was thought desirable to employ embryos of eggs of 5 or 6 days incubation, which had been used successfully for the cultivation of rabies-virus.<sup>3</sup>

Parallel cultures were set up, employing 1,000 chick cerebral-test-doses of A. E. virus, one group being inoculated by way of the yolk-sac, after the manner of Cox,<sup>4</sup> another, through an opening over the embryo, directly into the allantois. The eggs were then incubated at 36.5°C for 6-10 days, when their contents were removed and passage made from the allantoic membrane, yolk-sac, embryonic tissue, and brain to new series of eggs. The different tissues of the 2d and 4th passages were ground in broth and decimal dilutions of  $10^{-2}$  to  $10^{-7}$  or  $10^{-8}$  were injected intracerebrally into chicks. Five series of cultures were made and tested in 175 chicks, and in no instance could viral multiplication be discerned. In 2 instances, the brains of chicks hatched from such inoculated embryos were passaged in young chicks with negative results. Pathological examination of the CNS of embryos 18-20 days old (i.e., 12-14 days after inoculation), or of hatched chicks failed to reveal the characteristic lesions of encephalomyelitis.

In addition, tests were made on the survival of virus injected into young and older embryos. In the former, 21 eggs with 5- to 7-day-old embryos, and in the latter 11 eggs with 11- to 13-day-old ones were used, 0.1 cc of  $10^{-1}$  dilution of virus (active in  $10^{-6}$  dilution) was inoculated into the eggs through the allantois in the region of the embryo. The presence of virus in the embryos was demonstrated by pooling 2 or 3 inoculated embryos 1 hour later and injecting decimal dilutions of the suspension into chicks. After incubation at 37°C for varying periods, the embryonic brain and the other tissues were again examined for virus-content. In the series of

<sup>2</sup> Van Roekel, H., Bullis, K. L., and Clarke, M. K., *Vet. Med.* 1939, **34**, No. 12.

<sup>3</sup> Kligler, I. J., and Bernkopf, H., *Nature*, 1939, **143**, 899.

<sup>4</sup> Cox, H. R., *Pub. Health Rep.*, 1938, **53**, 2241.

young embryos, virus was recovered in dilutions up to  $10^{-3}$  after 24, 48, and 72 hours but not at all after 5 or 9 days, in the older ones, virus was recovered in a dilution of  $10^{-1}$  after 48 hours, but not after 8 and 11 days. Pathological examination of the brain and cord of these embryos 11 days after inoculation failed to show any characteristic lesions.

Finally 21 of the birds which had hatched out of inoculated eggs or had received intracerebral inoculation of the mentioned embryonic material and failed to show signs of the malady for at least 40 days, were then subjected to an intracerebral test for immunity. All came down with characteristic A/E virus infection, thus indicating the absence of active multiplying virus in the chicks and in the materials used in an amount sufficient to induce immunity.<sup>1</sup> At the time of the test, the fowls were 9-10 weeks of age.

2 *In Minced Embryonic Tissues in vitro* Three series of cultures of this type were set up. In one, minced chick-embryo brain plus Tyrode's solution and 10% chicken serum served as medium. The second series was the same as the first, except that minced whole chick embryo was used in place of brain. In the last Rivers' medium was used minced chick embryo in Tyrode's solution. In each instance the viral suspension as well as 2 flasks of the inoculated culture, was titrated for determination of the viral titer at the outset (Similar titrations of 2 or 3 culture flasks were carried out at the time of transfer). The cultures were incubated at 36.5 to 37°C and transfers into the respective media were made at 5-6 days' intervals. Each series was carried through 5 passages, so as to exclude any possible dilution factor of the original viral inoculum. The results are summarized in Table I and reveal that in the culture of whole-embryo tissue plus serum, there was a viral titer of  $10^{-3}$  at the outset and at least  $10^{-2}$  in the 5th subplant, although the original virus itself was calculated as being diluted to  $10^{-8}$  in this passage. In the other 2 series the virus was detectable only through the 2d subplant, this corresponds to the point beyond which the virus is diluted out of its original activity.

Again it is clear that the avian virus has distinctive requirements for multiplication such as are not found to exist for several others. Multiplication took place in minced whole embryos suspended in serum-Tyrode-solution mixture. The brain cultures were negative after the 2d subplant, as were those made with minced embryonic tissue in Tyrode's solution without serum.

It would appear therefore that the virus is rapidly lost at 37°C unless embryonic tissue and serum are present.

TABLE I  
Viral Activity in Various Minced Chick Embryo Media

Titer of stock virus used for seeding cultures	Media	Titer of virus + media immediately after set up	Subplants				
			1st	2d	3d	4th	5th
10 <sup>-3</sup>	Brain + serum	10 <sup>-3</sup>	10 <sup>-3</sup>	<10 <sup>-3</sup>	0	0	0
10 <sup>-5</sup>	Embryo + serum	10 <sup>-3</sup>	>10 <sup>-4</sup>	10 <sup>-2</sup>	10 <sup>-4</sup>	10 <sup>-4</sup>	10 <sup>-2</sup>
—	Embryo + Tyrode's solution	10 <sup>-4</sup>	>10 <sup>-2</sup>	10 <sup>-3</sup>	0	0	0

*Summary and Discussion* 1 Embryonic chicks are apparently not susceptible to infection with this avian virus although birds just hatched are. This is a strikingly reversed state from that which prevails in the case of certain other viruses<sup>5</sup> which multiply readily in the undifferentiated tissues of the developing embryo but are inactive in the hatched chick. Whatever the reason may be a fact of epidemiological significance emerges: the disease-agent is probably not transmissible by way of the egg thus supporting the prior finding of Jones<sup>1</sup>.

2 The virus multiplied in minced whole-embryo tissue-cultures *in vitro* only under certain indicated conditions. The method in its present state however is not favorable for obtaining large yields of highly potent virus for use in immunizing procedures. No multiplication of virus was noted in this medium when chick-embryo brain was used instead of whole-embryo tissue. It is of interest in this connection that mammalian embryo-brain cultures have been found suitable for the multiplication of the neurotropic viruses of poliomyelitis<sup>6</sup> and of rabies<sup>7</sup>.

<sup>5</sup> For a discussion and references, see Rivers, T. M. and Schwentker, F. F., *J. Exp. Med.* 1932, **55**, 911, and MacKenzie, R. D., *J. Path. and Bact.*, 1933, **37**, 75.

<sup>6</sup> Sabin, A. B., and Olitsky, P. K., *Proc. Soc. Exp. Biol. and Med.*, 1936, **34**, 357.

<sup>7</sup> Kanazawa, K., *Japanese J. Exp. Med.*, 1936, **14**, 519. Webster, L. T., and Clow, A. D., *J. Exp. Med.* 1937, **66**, 125, Bernkopf, H., and Khigler, L. J., *Brit. J. Exp. Path.*, 1937, **18**, 481.

# "Spreading" Properties and Mucolytic Activity of Leech Extracts

ALBERT CLAUDE.

*From the Rockefeller Institute for Medical Research, New York City*

In addition to their well known anticoagulating properties, leech extracts have the power to increase skin permeability<sup>1</sup>. Chemical studies from this laboratory have shown that the so-called spreading or "Reynals" factors, either from testicle,<sup>2</sup> or from the leech, were proteins<sup>3</sup>. Recently Chain and Duthrie<sup>4</sup> found that testicular extract would destroy the viscosity of the synovial fluid, and they have shown that this effect was due to the action of an enzyme on a polysaccharide. These authors suggested that this "mucnase" and the testicular spreading factor might be identical. If this is correct, one may expect leech extracts to have a high mucolytic activity, since their spreading power is 50 to 100 times greater than that of testicular extracts. In preliminary tests it was found that leech extract would destroy the viscosity of chicken tumor I extracts<sup>5</sup> with a remarkable speed, even at room temperature.

In the following experiments, the spreading power of various leech extracts was compared with their mucolytic activity, as measured by their action on chicken tumor I mucin.

*Effect of Leech Extracts on Chicken Tumor I mucin* Previous work has shown that the spreading factor is located in the anterior part of the leech (head)<sup>1</sup>. It is assumed that the substance is produced by the pharyngeal epithelium and plays a role in the process of feeding. On the other hand, a similar extract from the rest of the body is found to contain very little of the same factor.

In the present experiments separated heads and bodies were extracted in the usual way,<sup>1</sup> with 10 to 20 times their own weight of distilled water or phosphate buffer at pH 7.0 and centrifuged at 18,000 times gravity for 2 hours. These extracts were used as the source of enzyme. The substrates for mucolytic activity were concentrated extracts of chicken tumor I desiccates,<sup>5</sup> purified by high

<sup>1</sup> Claude, A., *J. Exp. Med.*, 1937, **60**, 353.

<sup>2</sup> Duran Reynals, F., *J. Exp. Med.*, 1929, **50**, 327, Hoffman D. C., and Duran Reynals, F., *Science*, 1930, **72**, 508, *J. Exp. Med.*, 1931, **53**, 387. McClean, D. *J. Path. and Bact.*, 1930, **33**, 1045.

<sup>3</sup> Claude, A., and Duran Reynals, F., *J. Exp. Med.*, 1937, **65**, 661.

<sup>4</sup> Chain, E., and Duthrie, E. S., *Nature*, 1939, **144**, 977.

<sup>5</sup> Claude, A., *J. Exp. Med.*, 1935, **61**, 27.

speed centrifugation The speed of the enzymatic reaction was studied at a temperature of 25°C

Under these conditions the leech head extract, in the concentration of 1%, by volume, was found to have a powerful effect on the chicken tumor mucin, bringing the relative viscosity of the extract from 7.7 to 1.5 in 5 to 10 minutes At that concentration, 30 minutes of contact with the leech factor brought the viscosity of the fluid close to that of water, the reduction in viscosity corresponding to 99.25% of the original value Leech head extract, in a final concentration of 0.01% reduced the original viscosity by 73.6% whereas the leech body extract, in a final concentration of 1%, brought about a reduction in viscosity of 73.9% This indicates that a 0.01% head extract and a 1% body extract have about equal strength, the former being then about 100 times more active than the latter

The spreading power of the leech extracts was tested by the injection of 10-fold dilutions of the solutions mixed with India ink, in the rabbit skin<sup>2, 1</sup> The head extract, at a dilution of  $10^{-3}$ , gave an area of spread equal to 20.3 sq cm, as compared with 22.6 sq cm for the body extract, at  $10^{-1}$  dilution This would indicate that the head extract is about 100 times more active than the body extract The above results are illustrated in Table I

The close parallelism between the rate of action of the mucinase and the spreading power of different extracts is strong evidence that the two factors in the leech are identical \*

This view is also supported by the fact that bull testicular extract was about 100 times less active than the leech extract, as regard both spreading power and mucolytic activity

*Mechanism of Spread* No satisfactory explanation has been found to account for the phenomenon of spread<sup>1</sup> The spread considered as the result of the mucolytic activity of the spreading factor would assume the presence, in the skin, of a chemically suitable substrate for the mucinase to act upon From the histochemical studies of Bensley<sup>6</sup> and Sylvén<sup>7</sup> it appears that normal or pathological tissues contain a viscid ground substance which, from its staining properties, resembles mucin

In the present work freshly removed rabbit skin was passed through a meat grinder and extracted by contact with 2 volumes of a 10% NaCl solution at 2°C for 24 hours This extract was filtered

\* The present work has been confined to the study of changes in viscosity under the effect of leech extracts assuming that the spreading phenomenon is brought about by similar changes in the skin without requiring necessarily complete hydrolysis of the substrate

<sup>6</sup> Bensley, S. H., *Anat. Rec.*, 1934, **60**, 93

<sup>7</sup> Sylvén, B., *Lunds Arch. Path. Anat.*, 1938, **303**, 280



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<sup>3</sup> Claude, A., and Duran Reynals, F., *J. Exp. Med.*, 1937, **65**, 661.

<sup>4</sup> Chain, E., and Duthrie, E. S., *Nature*, 1939, **144**, 977.

<sup>5</sup> Claude, A., *J. Exp. Med.*, 1935, **61**, 27.

Mucolytic Activity of Leech Extract on Normal Rabbit Skin Mucoprotein

Tests	Mucoprotein solution		Leech extract (head)		Mucolytic activity			
	Solids in sol mg per cc	Relative viscosity of sol	Amt added to mucoprotein solution		10 min at 25°C		30 hr at 25°C	
			By volume, %	Final conc in mixture, %	Relative viscosity of mixtures	Reduction in viscosity %	Relative viscosity of mixtures	Reduction in viscosity %
I	0.28	1.44	0.74	0.003	1.12	72.7	1.12	73.1
II	0.70	2.2	1.0	0.004	1.38	69.1	1.80	67.5
III	0.70	2.2	1.0	0.004	1.41	64.0		
IV	1.10	3.16	1.0	0.004	1.46	61.7		
					2.05	57.3		

TABLE I  
"Spreading" Property and Mucolytic Activity of Leech Extracts

Leech head extract									
Spread in rabbit skin					Mucolytic activity				
Amt solids injected, g	Area of spread, cm <sup>2</sup>	Ratio active spread to spread of control	Cone leech extr in mucin sol (by vol), %		Reduction viscosity after 30 min at 25°C, %		Leech body extract		
			Area of spread, cm <sup>2</sup>		Amt solids injected, g		Ratio active spread to spread of control		Reduction viscosity after 30 min at 25°C, %
3.6 x 10 <sup>-3</sup>	91.0	16.0	1.00	99.3	2.35 x 10 <sup>-3</sup>	66.9	11.7	1.0	73.9
3.6 x 10 <sup>-4</sup>	38.0	6.6	0.10	97.9	2.35 x 10 <sup>-4</sup>	22.6	4.0	0.1	35.2
3.6 x 10 <sup>-5</sup>	31.0	5.4	0.01	73.6	2.35 x 10 <sup>-5</sup>	8.0	1.4		
3.6 x 10 <sup>-6</sup>	20.3	3.2			2.35 x 10 <sup>-6</sup>	6.3	1.0		
3.6 x 10 <sup>-7</sup>	6.8	1.2			2.35 x 10 <sup>-7</sup>	7.0	1.0		
3.6 x 10 <sup>-8</sup>	8.0	1.4			2.35 x 10 <sup>-8</sup>	7.5	1.3		
3.6 x 10 <sup>-9</sup>	5.5	1.0			2.35 x 10 <sup>-9</sup>	6.2	1.0		

from the rest of the leech body. A comparable quantitative relationship is found to exist between head and body extracts when tested for another property, *e. g.*, their power to spread in the rabbit skin. 3 The parallelism in the strength of various extracts, as regards both mucolytic activity and spreading power, supports the view that the "mucinaase" and the leech spreading factor may be identical. 4 A mucoprotein has been prepared from normal rabbit skin. 5 The viscosity of a skin mucoprotein solution is rapidly and considerably reduced by the action of leech extracts. 6 The effect of leech extracts on the skin mucoprotein *in vitro* suggests that their ability to spread through the skin may be due, at least in part, to their power to cause hydrolysis or depolymerisation of the same or a similar compound *in vivo*.

## 11304

## Effect of Synthetic Vitamin K Compounds on Prothrombin Concentration in Man

P. M. AGGELER, S. P. LUCIA AND LEON GOLDMAN

From the Departments of Medicine and Surgery, University of California Medical School, San Francisco, California \*

The effectiveness of preparations of vitamin K in correcting hypoprothrombinemia has been demonstrated by numerous investigators.<sup>1</sup> Following the discovery of the antihemorrhagic activity of certain naphthoquinones,<sup>2</sup> several synthetic crystalline compounds were made available for clinical use in man. Butt *et al.*,<sup>3,4</sup> administered 2-methyl-3-hydroxy-1,4-naphthoquinone (phthiocol) in doses of 25 to 50 mg intravenously to 9 patients with obstructive jaundice or disease of the liver and 1,4-dihydroxy-2-methyl-naphthaldehyde in doses of 5 to 10 mg intravenously to 10 patients with obstructive jaundice. These preparations were effective in shortening the prolonged prothrombin time as calculated by the method of Quick. No untoward reactions were observed.

\* Assisted by a grant from the Christine Breon Fund.

<sup>1</sup> Quick, A. J., *Am J Med Sci*, 1940, **100**, 118.

<sup>2</sup> Almquist, H. J. and Klose, A. A., *J Am Chem Soc*, 1939, **61**, 1293.

<sup>3</sup> Butt, H. R., Snell, A. M., and Osterberg, A. E., *Proc Staff Meet Mayo Clin*, 1939, **14**, 497.

<sup>4</sup> Snell, A. M., and Butt, H. R., *J A M A*, 1939, **113**, 2056, footnote 41.

through paper and the filtrate treated with chloroform, with occasional shaking, for another 24 hours. Denatured proteins were discarded by centrifugation. The clear solution was then dialyzed in cellophane bags until free of NaCl. A protein fraction, different from the mucoprotein, separated out on dialysis, and was discarded by filtration through paper. All the operations were conducted in the cold, to avoid the action of a mucolytic enzyme which might have been extracted from the skin, together with the mucoprotein.

The skin mucoprotein is readily soluble in water, giving clear, slightly yellow solutions. The biuret reaction is positive. Solutions containing 0.7 mg solids per cc gave a strongly positive orcinol test. Like chicken tumor I mucin, it gives a stringy precipitate with neutral red. The behavior of the substance in acidic solution is noteworthy. Its solubility decreases progressively down to pH 4.0, giving a flocculent precipitate. At pH 4.0 there is a sudden change in the appearance of the precipitate which is then mucoid and contracts. Below pH 4.0 the protein becomes more soluble but the precipitate retains its mucoid character. Below pH 2.0 the substance is completely soluble. On account of the physical change taking place at that point, it is uncertain whether pH 4.0 is the point of minimum solubility of the protein.<sup>†</sup>

A solution containing 1.4 mg skin mucoprotein per cc had a relative viscosity of 3.46 at 25°C. Addition of leech extract to a final concentration of 0.004% produced a sudden drop in the viscosity of the solution. As a result of 5 different tests, it appears that leech extracts at the above concentration and acting for 30 minutes at 25°C will reduce the original viscosity of the solution by 68.4%. A contact of 30 hours at the same temperature will show but a slight additional effect, the total reduction in viscosity amounting to 70.4% of the original value. These results are in agreement with the observations of Meyer and coworkers<sup>8</sup> who showed that only 69% of the synovial mucin was hydrolyzed by 45 hours' incubation with a pneumococcus enzyme, in contrast with 96% hydrolysis for the free polysaccharide, under the same conditions.

*Summary and Conclusions* 1. Leech extracts contain a powerful mucolytic enzyme, as shown by its effect on the viscosity of chicken tumor I extracts. 2. Leech head extracts exhibit a mucolytic activity considerably greater than that of similar extracts obtained

<sup>†</sup> The name "mucoprotein" refers especially to the physical properties of the material since the solution may contain different soluble components of the skin.

<sup>8</sup> Meyers, K., Hobby, G. L., Chaffee, E., and Dawson, M. H., *J. Exp. Med.* 1940



We have studied the effectiveness of 3 synthetic naphthoquinones—2-methyl-3-hydroxy-1,4-naphthoquinone (phthiocol) 2-methyl-1,4-naphthoquinone and 4-amino-2-methyl-naphthol-hydrochloride—in 26 patients 11 having obstructive jaundice 11 chronic diseases of the liver 2 acute diseases of the liver 1 non-tropical sprue, and 1 gastro-colic fistula The prothrombin concentration was determined by the method of Quick\* All determinations were made in duplicate

Phthiocol† was administered intravenously in doses of 50 to 300 mg. of a 0.5% solution 4-amino-2-methyl-naphthol-hydrochloride‡ was administered intravenously in doses of 5 to 30 mg of a 0.1% solution Gelatin capsules containing 0.6 mg of 2-methyl-1,4-naphthoquinone§ dissolved in corn oil were administered orally in daily doses of 3.6 to 13.2 mg The total daily dose was divided into 3 equal parts and given after meals With the exception of Case 25, 100 mg of desoxycholic acid was given with each 1.2 mg of 2-methyl-1,4-naphthoquinone Bile salts were not used in Case 25 nor given to those patients who received the intravenous preparations

#### *Results A Obstructive Jaundice (Table I)*

The prothrombin concentration before treatment varied from 3% to 45% and 24 hours after treatment from 60% to 130% The duration of the significant effect produced by the intravenous compounds lasted in some cases as long as 7 days The administration of 50 mg phthiocol (Case 10) elevated the prothrombin concentration to 50% in one hour, 60% in 4 hours and 100% in 24 hours After the intravenous administration of 10 mg of 4-amino-2-methyl-naphthol-hydrochloride (Case 6) the prothrombin concentration was elevated to 30% in 1 hour 50% in 4 hours 75% in 8 hours and 100% in 24 hours In Case 1 the prothrombin concentration was maintained at 100% for 3 months on a daily oral dose of 0.6 mg of 2-methyl-1,4-naphthoquinone The relatively poor response observed in Case 4 may have been due to a severe diarrhea from which this patient suffered In Case 6 following exploratory laparotomy a rapid diminution in the prothrombin concentration was observed

#### *B Chronic Diseases of the Liver (Table II)*

\* Quick, A. J., and Grossman, A. M., *PROC. SOC. EXP. BIOL. AND MED.*, 1939, **41**, 227

† Made available through the courtesy of the Galen Company, Berkeley, California

‡ Made available through the courtesy of Parke Davis and Co., Detroit, Michigan

The prothrombin concentration before treatment varied from 25% to 60% and 24 hours after treatment from 35% to 60%. The maximum prothrombin concentration noted in any patient during the 6-day period following the institution of treatment was 65%. There was no significant rise in the prothrombin concentration following treatment in 8 of the 11 patients studied. There was a slight elevation in the prothrombin concentration in 3 cases. These patients were moribund and died within 24 hours of the last prothrombin determination. Large single doses (Cases 20 and 22), and repeated standard daily doses (Case 21) of phthiocol did not elevate the prothrombin concentration beyond that accomplished with a single standard dose. In Case 12, 3.6 mg of 2-methyl-1,4-naphthoquinone was given orally daily for 1½ months, and no significant variation in the prothrombin concentration was observed.

*C Acute Diseases of the Liver* Case 23 (acute cholangitis, acute hepatitis, and *B. coli* septicemia) received oral daily doses of 3.6 mg of 2-methyl-1,4-naphthoquinone. The prothrombin concentration before treatment was 70% and on the following 6 days it was 65%, 50%, 75%, 80%, 80% and 90%. The relative refractivity and the nature of the response of this patient appeared to be conditioned by the severity of the illness. Case 24 (acute yellow atrophy of the liver) received 50 mg of phthiocol intravenously. The prothrombin concentration before treatment and 8 hours later was 20%. This patient died 12 hours after the last test.

*D Gastrointestinal Diseases* Case 25 (non-tropical sprue) received daily oral doses of 3.6 mg of 2-methyl-1,4-naphthoquinone. Bile salts were not given. The prothrombin concentration before treatment was 30%, and on the following 6 days it was 55%, 75%, 70%, 70%, 70% and 75%.

Case 26 (gastro-colic fistula) was treated with 4-amino-2-methyl-naphthol-hydrochloride and phthiocol. The prothrombin concentrations were as shown in Table III.

TABLE III

Days	Prothrombin conc, %	Days	Prothrombin conc, %	Days	Prothrombin conc, %
1*	50	9	60	21§	70
2	75	10	60	22	80
4	55	11	60	23	70
5†	60	12‡	70	25	85
6	55	13	70	28	70
7	75	14	70	36	65
8	75	19	60	43	55

\*Single intravenous dose of 10 mg 4-amino-2-methyl-naphthol hydrochloride

† " " " " 20 " 4-amino-2-methyl-naphthol hydrochloride

‡ " " " " 30 " 4-amino-2-methyl-naphthol hydrochloride

§ " " " " 30 " 4-amino-2-methyl-naphthol hydrochloride



TABLE II  
Chronic Liver Disease

Case No	Diagnosis	Compound administered	Dosage mg	Before treatment %	Prothrombin concentration									
					On day following institution of treatment									
					1 %	2 %	3 %	4 %	5 %	6 %				
12	Portal cirrhosis	2 methyl 1,4 naphthoquinone	13 2 daily	50	40	—	40	45	40	35				
13	" "	" "	5 4 "	45	35	40	—	—	—	35				
14	Banti's disease	" "	5 4 "	30	35	30	—	35	—	—				
15	Portal cirrhosis	4 amino 2 methyl naphthol HCl	30	50	50	—	50	—	—	50				
16	" "	" "	30	60	60	60	60	—	—	—				
17	" "	" "	30	25	50	65	60	50	50	—				
18	Primary carcinoma of the liver	" "	10	30	50	45	†	—	—	—				
19	Portal cirrhosis	Phthocol	50	35	35	40	—	50	55	65				
20*	" "	" "	50	40	45	45	45	50	40	40				
21	Primary carcinoma of the liver	" "	300	45	40	35	—	45	—	40				
21†	" "	" "	50	40	55	—	55	—	65	—				
22	Portal cirrhosis	" "	50 daily	55	50	65	50	†	—	—				
			50	55	55	—	55	55	50	—				
			followed by 150 mg on third day											

\*21 days after first injection

†11 " " "

†Patient died

while that of the chlorides rises. With passing off of the pituitary effect the concentrations return to or toward their original levels. In a consideration of these changes the following view was expressed: "It is possible that with these high rates of filtration the maximum absorbing capacity of the tubules is approached, so that changes in filtration from various experimental procedures can be observed in a relatively uncomplicated form."<sup>1</sup>

In the previous report no estimates were made of the changes in osmotic concentration of the urine during the changes in filtration. In the present study changes in depression of the freezing point have been used to indicate changes in osmotic concentration. The finding that these values are relatively constant has been taken as supporting the suggestion of the earlier report, namely that under the conditions of experiment the maximum concentrating capacity of the tubules is approached.

*Experimental.* Large rabbits, under urethane anesthesia were given 10% sucrose solution intravenously by means of the intravenous infusion apparatus recently described from this laboratory.<sup>2</sup> Urine was collected into graduated centrifuge tubes for 5-minute intervals. The rate of secretion always exceeded one cubic centimeter per minute. Chlorides were determined by a modified Volhard-Arnold method, specific gravity by weighing in a one cubic centimeter pycnometer, depression of freezing point using a Beck-

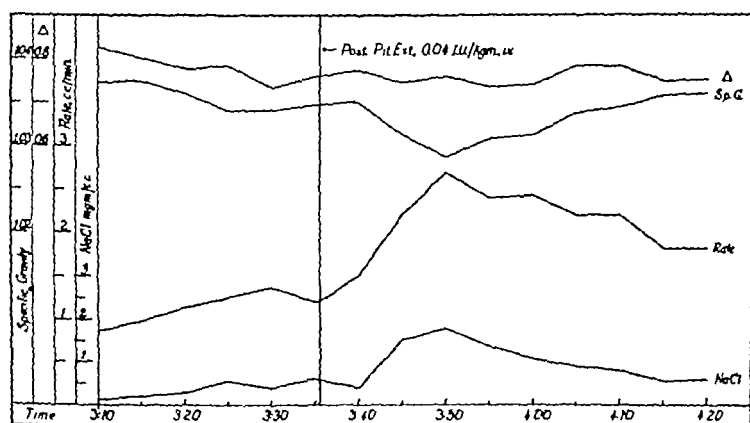


FIG 1

Effect of injection of posterior pituitary extract on rate, chloride content, specific gravity, and freezing point depression, of urine formed during continuous intravenous infusion of 10% sucrose solution, in a rabbit. Morphine urethane anesthesia.

*Summary* Three synthetic vitamin K compounds were administered to 26 patients with hypoprothrombinemia. Following treatment the prothrombin concentration (1) was markedly elevated in 11 patients with obstructive jaundice, (2) was not elevated in 8 and only slightly elevated in 3 patients with chronic diseases of the liver, (3) was not elevated in 1 patient and after an initial delay was elevated in 1 patient with acute diseases of the liver, (4) was moderately elevated in 2 patients one with non-tropical sprue, and the other with gastro-colic fistula. When considered in terms of the dosages employed, there were no significant qualitative differences in the relative effectiveness of the three compounds. No untoward reactions were observed except that the patients receiving large doses of 4-amino-2-methyl-naphthol-hydrochloride complained of slight burning pain at the site of injection.

## 11305 P

### Effect of Posterior Pituitary Extract on Concentration of Urine Secreted during Osmotic Diuresis in Rabbit \*

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When 10% sucrose is infused rapidly into the circulation of anesthetized rabbits, there follows the rapid elimination of a large amount of urine having a sugar concentration approaching that of the infusion fluid, and almost keeping pace with it as to rate. The injection of posterior pituitary extract during this diuresis results in a further acceleration of rate. This has been shown to be due to an increase in glomerular filtration<sup>1</sup>. If from the calculated filtration using the amount of sucrose in plasma and urine for making the calculation, there be subtracted the amount of urine actually eliminated, there is obtained a figure which represents the amount of filtrate reabsorbed during the passage through the tubules. During this phase of increase in filtration from pituitary extract there not only is no decrease in absorption but there may actually be a slight increase. During this pituitary "diuresis" the sugar content of urine falls.

\* Supported in part by Grant No. 315 from the Council on Pharmacy and Chemistry of the American Medical Association.

<sup>1</sup> Nelson, E. E., *J. Pharm. Exp. Therap.* 1934, **52**, 194.

In this communication we wish to report the finding of a perfusate of ischemic kidneys which causes the elevation of blood pressure following the release of complete renal ischemia. Until the precise nature of the active principle has been determined, we propose the name *ischemin*, which may be defined as the pressor substance contained in perfusates of completely ischemic kidneys and which is responsible for the hypertension resulting from the release of total renal ischemia. For convenience, the renal perfusate containing the pressor substance will also be termed *ischemin*.

The results of the observations made thus far are summarized briefly.

In 8 experiments, cats were anesthetized with ether and the renal pedicle of one kidney clamped. After 4 to 6 hours the animal was reanesthetized with nembutal, the blood pressure recorded from the carotid artery and both the normal and ischemic kidneys removed. Within 5 minutes following the removal of the kidneys, both renal arteries were cannulated and the kidneys perfused with 1 cc of warm saline per gram of kidney. The perfusates were then injected intravenously into the same animal. The perfusates of the ischemic kidneys uniformly gave a marked and prolonged rise in blood pressure, amounting to as much as 100 mm of Hg, whereas those obtained from the normal kidneys had no pressor effect.

Perfusates of ischemic hind limbs of cats were found to contain no pressor substance.

The first series of experiments having demonstrated the presence of pressor material (*ischemin*) in perfusates of ischemic kidneys it was decided to determine whether this substance was responsible for the hypertension resulting from the release of complete renal ischemia (Taqui<sup>1,2</sup>). If *ischemin* causes this rise in blood pressure, there would necessarily be less *ischemin* in the perfusate of the kidney, the circulation of which has been reestablished than in a control ischemic kidney in which the circulation has not been reestablished. For this reason the following experiment was devised.

Both renal pedicles of 22 cats were clamped under ether anesthesia. After 4 to 6 hours the animals were reanesthetized with nembutal and the blood pressure recorded from the carotid artery. In each instance, upon reestablishing the circulation of one kidney, the usual rise in blood pressure took place.<sup>2</sup> The other kidney was not unclamped. Fifteen minutes later both kidneys were removed and perfused in the manner previously described. The perfusate of the released kidney was injected intravenously into the same animal followed after a suitable interval by the perfusate of the unreleased kidney.

man thermometer in the usual manner. When the rate had been constant for at least 4 5-minute periods posterior pituitary extract was given intravenously, in amounts of the order of 0.04 International Units per kg body weight.

As in the previous study, this amount of pituitary was followed almost invariably by an increase in both rate and chloride content. Sucrose determinations were not made, but in the previous series it invariably fell. Specific gravity either fell or remained constant, and the freezing point remained practically constant. The results, which were consistent throughout, are illustrated in the record of a single experiment shown in Fig. 1.

*Conclusions.* The finding that the osmotic concentration, as revealed by the depression of the freezing point, remains constant during the action of pituitary extract on the diuresis induced by rapid intravenous infusion of sucrose solutions, lends support to the view that the absorption capacity of the tubules has been reached under these conditions of experiment.

## 11306

### Etiology of Hypertension Due to Complete Renal Ischemia

MYRON PRINZMETAL \* HARVEY LEWIS AND SIDNEY LEO † (Introduced by Ella H. Fishberg)

*From the University of Southern California Medical School*

It is generally agreed that the hypertension which develops in experimental animals as a result of partial<sup>1</sup> or complete<sup>2</sup> ischemia of the kidneys is of humoral and not of nervous origin.

Although *renin*, a pressor extract prepared from the renal cortex, has been implicated by some observers<sup>3, 4</sup> neither this product nor any other has to date been proved to be the substance responsible for ischemic hypertension.

\* Dazian Fellow

† Beaumont Research Fellow

<sup>1</sup> Goldblatt, H., Lynch, J., Hanzal, R. F., and Summerville, W. W. *J. Exp. Med.* 1934, **59**, 347.

<sup>2</sup> Taquini, A. C., *Rev. Soc. argent. de biol.* 1938, **14**, 422.

<sup>3</sup> Prinzmetal, M., and Friedman, B. *Proc. Soc. Exp. Biol. and Med.* 1936, **35**, 122.

<sup>4</sup> Harrison, T. R., Blalock, A., and Mason, M. F. *Proc. Soc. Exp. Biol. and Med.* 1936, **35**, 38.

TABLE I

Time	Pressor effect
3 24	40 mm Hg
3 50	32
4 17	28
4 27	24
4 33	14
4 43	14
5 05	12

The amount of *renin* in extracts of normal, unreleased ischemic and released ischemic kidneys is now being investigated

*Ischemin* exhibits the following characteristics

a Tachyphylaxis By this is meant that repeated injections of a pressor substance into the same animal cause decreasing effects An example in the case of *ischemin* is shown in Table I

b The boiling of *ischemin* for 5 minutes destroys the active pressor principle

c The action of *ischemin* is not reversed by 933F (piperidomethyl-3-benzodioxane)

From these experiments it is concluded that as a result of complete renal ischemia pressor material (*ischemin*) accumulates in the kidney and is readily washed out into the general circulation when the renal blood flow is reestablished or by artificial perfusion of the extirpated organ This is the first time a pressor substance has been obtained which has been proved to be the cause of hypertension due to renal ischemia

This substance (*ischemin*) has certain properties in common with *renin* destruction by heat similar character of the pressor curves the property of tachyphylaxis and failure of 933F to abolish the pressor reaction<sup>a</sup> but until decisive physiological and chemical studies have been performed no definite statement should be made concerning the relationship of these two products, nor to the heat-stable renal pressor substance which has recently been described<sup>c</sup>

<sup>a</sup> Kritz, L. N., and Friedberg, L., *Am J Physiol.*, 1939, **127**, 27

<sup>c</sup> Victor, J., Steiner, A., and Weeks, D. M., *Proc Soc Exp Biol and Med.*, 1939, **42**, 767

It was found that the perfusate of the unreleased kidney showed a significantly greater pressor effect than that of the released kidney, the average rise in blood pressure being 33 mm and 16 mm of Hg respectively

The smaller pressor response of the perfusate of the released kidney, as compared with that of the unreleased kidney, is readily explained by the washing out into the general circulation of some (or all) of the *ischemin* from the released kidney upon removal of the clamp

The perfusate of the released kidney was intentionally injected first because of the phenomenon of tachyphylaxis (see *ischemin* characteristics a) Except for this phenomenon, the difference in pressor response would have been considerably greater

Additional evidence that *ischemin* is the substance responsible for the hypertension occurring after reestablishment of the circulation of completely ischemic kidneys follows

1 933Γ (piperidomethyl-3-benzodioxane) does not reverse the rise in blood pressure following reestablishment of the circulation of completely ischemic kidneys or the pressor response to *ischemin*

2 The blood pressure curve following reestablishment of the circulation of completely ischemic kidneys is similar to that produced by intravenous injection of *ischemin*

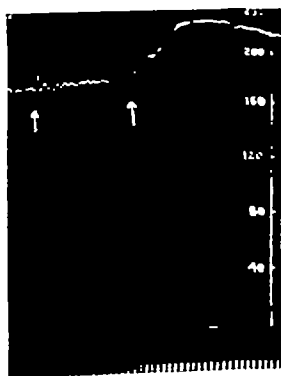


FIG A

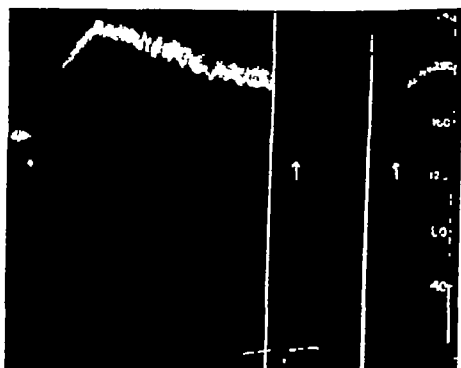


FIG B

At first arrow, intravenous injection of perfusate of normal right cat's kidney  
At second arrow, injection of perfusate of same animal's left kidney rendered completely ischemic for 5 hours

FIG B

Five hours previously, both renal pedicles clamped At first arrow, clamp removed from left renal pedicle At second arrow, intravenous injection of perfusate of left (released) kidney At third arrow, injection of perfusate of right (unreleased) kidney

Nembutal anesthesia Time 15 second intervals

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This substance (*ischemin*) has certain properties in common with *renin*: destruction by heat; similar character of the pressor curves, the property of tachyphylaxis and failure of 933F to abolish the pressor reaction<sup>a</sup> but until decisive physiological and chemical studies have been performed, no definite statement should be made concerning the relationship of these two products, nor to the heat-stable renal pressor substance which has recently been described<sup>b</sup>

<sup>a</sup> Katz, L. N., and Friedberg, L., *Am J Physiol*, 1939, **127**, 27

<sup>b</sup> Victor J., Steiner, A., and Weeks, D. M., *Proc Soc Exp Biol and Med*, 1939, **42**, 767



# Method for Obtaining Group A Hemolytic Streptococci Sensitive to Reaction of Capsular Swelling

A RINFRET (Introduced by Arthur F Coca)

From the Research Department of Lederle Laboratories, Inc., Pearl River, New York

The following experiments are an approach to the problem of mouse virulence and type-specificity of Lancefield Group A beta hemolytic streptococci from the standpoint of encapsulation and the reaction of capsular swelling with homologous serum. Many of our stock cultures were avirulent with little or no demonstrable capsules. Attempts to produce capsular swelling with these organisms were unavailing. Further, although capsules were at times noted on our most virulent cultures, all attempts to produce capsular swelling were negative.

It has been shown by Dawson,<sup>1</sup> Seastone,<sup>2</sup> Loewenthal<sup>3</sup> and others that encapsulation of hemolytic streptococci is an attribute always accompanying highly virulent invasive strains. Loewenthal<sup>3</sup> regarding the encapsulated organism as capable of evoking protective antibodies used only this type of organism in producing protective antisera. He succeeded in making antisera from mucoid and non-mucoid strains, but in either case he emphasized the presence of capsules. In an earlier paper<sup>4</sup> he noted the "swelling" of these capsules with the addition of homologous serum to the organisms.\*

The logical approach to the problem of increasing virulence is by mouse passage. For our purposes this method offered certain difficulties. Parish and Okell,<sup>5</sup> Pulvertaft,<sup>6</sup> and Hartley<sup>7</sup> have shown that streptococci elaborate toxins which are lethal for mice. Todd<sup>8</sup> further showed a limited protection with an antitoxin (antistreptolysin O) against living cultures of streptococci injected into mice. From these experiments it is clear that toxemia may in large meas-

<sup>1</sup> Dawson, M. H., Hobby, G. L., and Olmstead, M., *J. Inf. Dis.* 1938, **62**, 138.

<sup>2</sup> Seastone, C. V., *J. Exp. Med.*, 1939, **70**, 347.

<sup>3</sup> Loewenthal, H., *Brit. J. Exp. Path.*, 1938, **19**, 143.

<sup>4</sup> Loewenthal, H., *Brit. J. Exp. Path.*, 1934, **15**, 298.

\* It should be noted that these organisms fall serologically into Lancefield's

## Group B

<sup>5</sup> Parish, H. J., and Okell, C. C., *J. Path. Bact.* 1927, **30**, 521.

<sup>6</sup> Pulvertaft, R. J. V., *J. Path. Bact.* 1928, **9**, 276.

<sup>7</sup> Hartley, P., *Brit. J. Exp. Path.*, 1928, **9**, 259.

<sup>8</sup> Todd, E. W., *Brit. J. Exp. Path.* 1938, **19**, 367.

ure, be responsible for the deaths of mice injected with living streptococci

Believing that a death for which a septicemia was entirely responsible would yield an organism better suited to our purpose, scarlet fever antitoxin was injected simultaneously with culture into the mouse peritoneum. It was thought that the inhibition of toxin production or the neutralization of toxin as quickly as it was formed would accomplish the desired result.

*Procedures* 1 *Mouse Passage* Thirteen strains originally obtained from Griffith and representing 13 serological types in his classification (types 1, 2, 3, 9, 10, 12, 13, 14, 15, 23, 25, 26, 28) were used. Cultures were taken from vacuum tubes planted in beef-infusion broth with 10% of normal horse serum and incubated at 37° for 18 hours. Five-tenths of a cubic centimeter of the culture, in each case, was inoculated into another tube of serum broth and incubated at 37°C for 4 hours, 0.5 cc of the dilution containing approximately one lethal dose was injected simultaneously with 0.5 cc of unrefined scarlet fever antitoxin† into a mouse. The intraabdominal route was used.

Immediately upon the death of the mouse (5 to 24 hours) the spleen was excised aseptically and macerated in 1 cc of sterile peptone water. To one-half cc of this suspension, one-half cc of antitoxin was added. This mixture was immediately reinjected into mice.

After excision of the spleen the heart's blood was inoculated into serum broth. When growth was sufficient, a capsular swelling preparation was made and the reaction noted.

Upon the death of the mice injected with the splenic material, the culture was again tested for capsular swelling. When positive reactions were consistently obtained, the cultures were put under vacuum and stored at 4°C.

2 *Action on horse blood agar* Stock cultures and antitoxin-treated cultures were plated and any variations noted. The stock culture plate served as the control.

3 *Growth in serum broth* Cultures were grown in serum broth for varying periods of time at 37°C and room temperature (1 to 72 hours) the macroscopic and microscopic conditions of the growth were noted. Microscopic examination was made primarily to ascertain the presence or absence of capsules.

4 *Capsular swelling preparations*

a 1 mm loop of culture

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† 200 to 500 units U.S.P.H.S. or 10,000 to 25,000 original neutralizing units

- b 5 mm loop of antiserum
- c 5 mm loop of methylene blue
- d Incubation at 52° for varying periods of time ( $\frac{1}{2}$  to 24 hrs)
- e Control with saline or with heterologous serum for the detection of cross reactions

**Results** The results of these experiments may best be shown by a comparison with beta hemolytic streptococci not so treated. The treated organisms generally show encapsulation which is readily accentuated by the presence of specific antiserum. The virulence of the treated organism is increased and the hemolytic zone around the colonies is altered. These results are shown in Table I.

**Summary** A method of obtaining virulent, encapsulated group A beta hemolytic streptococci, sensitive to the reaction of capsular swelling with homologous antisera, has been described. Essentially this consists of the injection of living streptococci into mice simultaneously treated with unrefined scarlet fever antitoxin.

TABLE I

Untreated	Treated with Antitoxin
1 Action on horse blood agar	
a Hemolytic zone sharply defined and transparent	a Hemolytic zone frequently not sharply defined and is generally translucent (18 to 24 hours)
b No pigment was ever noted on base of colony	b Base of colony frequently showing rose to deep rose shades
c Smooth or rough colonies	c Smooth colonies
2 Growth in broth	
a Often flocculent with sediment	a Invariably of even turbidity
b Capsule not visible in ordinary wet methylene blue preparation	b Capsule frequently visible in wet methylene blue preparation.
c Soon loses capsules in broth as shown by dry capsule stains	c Capsules observed with capsule stains after 48 hours in many cases
3 Capsular swelling	
a Negative with specific antiserum	a Positive with antiserum. This reaction is unmistakable
4. Agglutination.	
a Organisms readily agglutinated with homologous serum using rapid slide agglutination test	a To date these organisms have proved inagglutinable with homologous serum, using rapid slide test
5 Virulence for mice	
a May or may not be virulent	a Virulent (10 <sup>-4</sup> to 10 <sup>-9</sup> )
b If virulent, 10 lethal doses generally kill in 16 to 48 hr	b 10 lethal doses kill in 5 to 12 hr
c Many mouse passages are necessary to build virulent strains.	c With strains so far employed a relatively few passages (1 to 10) are required to obtain the desired virulence

## 11308 P

## Origin of L Type Colonies in Bacterial Cultures \*†

L DIENES

*From the Departments of Pathology and Bacteriology, Massachusetts General Hospital, Boston, Mass*

Small secondary colonies corresponding morphologically to young colonies of the L1 strain isolated by Klieneberger from cultures of *Streptobacillus moniliformis*, have been observed occasionally in cultures of various Gram negative bacteria<sup>1</sup> In all cultures in which such secondary colonies developed a large number of bacteria swelled up into large deeply stained spherical or fusiform bodies It was first seen in a culture of *Bacillus influenzae* that occasionally L type of colonies developed from the large bodies Later this process was observed repeatedly in the cultures of 2 colon bacillus strains

After 24 hours of growth the colonies of these strains consisted mainly of large spherical bodies In order to observe the development of these large bodies in these 2 strains different procedures proved to be helpful The colonies of one strain were very tenacious and when an impression of the colonies was made on blood agar plates only the large bodies with very few bacteria, were transferred After 6 to 12 hours fine curved filaments grew from the large bodies into the agar and after 24 hours about two-thirds of them developed into tiny L type colonies With the other strain a similar process was observed when a broth culture was transferred on blood agar plates after varying time intervals In the transplant made after 24 hours' growth many large bodies were visible on the agar surface Only a few bacterial colonies developed in these plates but after 6 to 12 hours the large bodies began to germinate and produced L type colonies Transplants made from the broth culture after 48 hours, gave only abundant bacterial growth With either strain L type colonies developed only at the places where large bodies were present Therefore it is extremely unlikely that the L type colonies develop from invisible granules attached accidentally to the large bodies or from regularly shaped bacteria In transplants made with platinum loops from agar cultures the development

\* The expenses of this investigation have been defrayed, in part, by a grant from the Commonwealth Fund

† This is publication Number 43 of the Robert W Lovett Memorial Fund for the study of crippling disease, Harvard Medical School, Boston, Mass

<sup>1</sup> Dienes, L., PROC SOC EXP BIOL AND MED., 1939, **42**, 636

of the large bodies into the L type colonies was difficult to demonstrate because the agar was overgrown by bacteria

Subsequent to these observations it was found that the germination of the large bodies of *Streptobacillus moniliformis* could be observed if appropriate media were employed. The best results were obtained with an alkaline meat infusion agar (pH 8.2) without the addition of blood or ascitic fluid. A freshly isolated strain of *Streptobacillus moniliformis* was used. After 24 to 36 hours' growth on ascitic fluid media the colonies of this strain consisted mainly of large spherical bodies. On the alkaline agar macroscopic growth was observed only in thickly seeded areas. This growth consisted of regular *Streptobacilli* showing little pleomorphism. The remains of the abundant transplant degenerated and could not be stained. In the thinly seeded areas bacterial growth was absent but the large bodies remained well preserved and deeply stained. From a large number of them tiny L1 colonies were seen to grow in a manner similar to that noticed in the colon bacillus cultures. No L type colonies developed in the absence of the large bodies. Temperature between 25-30°C was most favorable for the germination of the large bodies.

According to these observations there are two stages in the development of L colonies. One first notes that the bacteria swell and form large deeply stained bodies. Under appropriate cultural conditions these germinate and produce the L type colonies. In cultures of L1 strains growth may be transmitted by small filter-passing elements. The transition from regular bacilli into the L form seems to occur through the large bodies. The significance of these tiny colonies, consisting often of very small elements, developing from the bacteria in this peculiar way is very puzzling. The L1 strains are antigenically similar to the *Streptobacillus moniliformis*<sup>2, 4</sup> and occasionally revert to the typical *Streptobacillus*<sup>2, 4</sup>. These studies leave no doubt that the L1 strain is a variant form of the *Streptobacillus* and not an extraneous element in the cultures. In the case of other bacteria we can infer only by analogy that they represent a variant form.

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<sup>2</sup> Dienes, L, *J Inf Dis*, 1939, **65**, 24.

<sup>3</sup> Klieneberger, E, *J Hyg*, 1938, **38**, 458.

<sup>4</sup> Dawson, M. H., and Hobby, G. L., *Transactions Assn Am Phys*, 1939 **44**, 329, Third International Congress for Microbiology, Abstracts of Communications, New York, 1939.

## 11309 P

## Reversibility of Quellung Phenomenon on Addition of Type-Specific Polysaccharide \*

W J NUNGESTER AND ALICE H KEMPF

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Although pneumococci can be typed directly from sputum it has been recognized that the results are sometimes unsatisfactory. Taplin, Meneely and Hettig<sup>1</sup> have outlined an improved method using fresh sputum to obtain the Quellung phenomenon. Essentially, this method consists of breaking up and suspending the sample in saline, centrifuging, and using the sediment for typing. The authors suggested that the water-soluble capsular polysaccharide might well be the interfering substance, which was removed by their technic.

In work published elsewhere,<sup>2</sup> it has been determined that in undiluted samples of ground lung from pneumonic rats, pneumococci present did not give the Quellung reaction with specific antiserum. The reaction did occur if the material were sufficiently diluted with PSS. Heidelberger<sup>3</sup> has shown that excess of antigen prevents visible evidence of antigen-antibody reaction, and has ably discussed the reversibility of the precipitin and agglutinin reactions. Since the above findings indicated that excess antigen prevented the occurrence of the Quellung reaction, it seemed that reversibility of this phenomenon might likewise take place. Hence, the following experiments were performed.

In each of 4 serological tubes were placed 0.1 cc of a broth culture of Type I pneumococcus and 0.1 cc of undiluted type-specific rabbit antiserum, after a short interval, the pneumococci were examined for the presence of swollen capsules. These were uniformly present. Then 0.1 cc of dilutions of Type I polysaccharides were added to each tube to give final amounts of 1.0 mg, 0.1 mg, 0.01 mg and 0.001 mg in 0.3 cc of fluid, respectively. Five minutes later, the preparations were re-examined. In the first 2 tubes, the swollen capsules had disappeared. In the last 2 tubes, no change was observed.

A similar reaction was seen employing Type III pneumococcus. However, the Type III specific polysaccharide was partially effective.

\* These studies were aided by a grant from the Horace H. Rackham School of Graduate Studies.

<sup>1</sup> Taplin, G. V., Meneely, G. R., and Hettig, R. A., *J. A. M. A.*, 1938, **111**, 410.

<sup>2</sup> Kempf, A. H., and Nungester, W. J., *J. Infect. Dis.* 1939, **65**, 1.

<sup>3</sup> Heidelberger, M., *Bact. Rev.* 1939, **3**, 49.

in causing this reversibility, when 0.01 or even 0.001 mg of the substance was added to the tubes containing previously swollen capsules. The specificity of this reversibility was indicated by the fact that addition of Type I polysaccharide failed to reverse the reaction.

## 11310

### Effect of Sulfapyridine, Sulfathiazole and Sulfamethylthiazole upon Severe Staphylococcal Infection in Mice \*

ELEANOR A. BLISS† AND EARL OTT (Introduced by P. H. Long)

From the Biological Division of the Department of Medicine, The Johns Hopkins University Medical School and The Johns Hopkins Hospital, Baltimore, Maryland

In previous reports<sup>1, 2</sup> it was shown that while sulfanilamide therapy had a slight effect upon the course of staphylococcal infections in mice, the use of sulfapyridine markedly prolonged the lives of the infected animals. In a few instances the mice treated with sulfapyridine survived 2 and 3 months.<sup>3</sup> Eventually, however, they succumbed showing at postmortem, abscess formation in the kidneys and liver.

Preliminary studies<sup>4, 5</sup> in which the *in vitro* effect of sulfathiazole was tested indicated that this compound inhibited the growth of a number of microorganisms, including staphylococci, to a greater degree than did sulfanilamide and sulfapyridine. It was then considered worthwhile to compare the activity of this compound and of sulfapyridine in the treatment of severe staphylococcal infections in mice. Later the activities of sulfathiazole and sulfamethylthiazole were similarly compared.

**Methods.** The mice were infected by the intravenous injection of heavy broth suspensions of a strain of *Staphylococcus aureus* which had been isolated a year previously from a patient, ill with a lung abscess. The method is given in detail elsewhere.<sup>1</sup>

\* This study was supported by the Chemical Foundation, Inc., of New York City.

† Fellow in Medicine, The Johns Hopkins University Medical School, on a grant from the Calco Division of the American Cyanamid Company.

<sup>1</sup> Feinstein, W. H., Bliss, E. A., Ott, E., and Long, P. H. *Bull. Johns Hopkins Hosp.*, 1938, **62**, 565.

<sup>2</sup> Bliss, E. A., and Long, P. H., *Proc. Soc. Exp. Biol. and Med.*, 1939, **40**, 32.

<sup>3</sup> Unpublished observations.

<sup>4</sup> Lawrence, C. A., *Proc. Soc. Exp. Biol. and Med.*, 1940, **43**, 92.

<sup>5</sup> Long, P. H., and Bliss, E. A., *Proc. Soc. Exp. Biol. and Med.*, 1940, **43**, 324.

TABLE I.  
The Effect of Diets Containing 1% Sulfapyridine, 1% Sulfathiazole or 1% Sulfamethylthiazole upon the Survival Time of Mice Infected with *Staphylococcus aureus*

Day of experiment	*Series A			†Series B			Series A		Series B	
	No of mice			No of mice			No of mice		No of mice	
	30	49	50	19	30	30	20	20	30	30
	Controls	Sulfapyridine	Sulfathiazole	Controls	Sulfathiazole	Sulfamethylthiazole	Sulfapyridine	Sulfathiazole	Sulfathiazole	Sulfamethylthiazole
	No of mice dying						Avg daily consumption of drug—mg/mouse			
-2	(before infection)						not measured		20.2	18.9
-1							22.5	22.4	26.6	24.7
1	14	11	4	3	0	0	6.5	14.6	14.2	14.3
2	1	3	4	0	2	1	21.7	31.6	23.5	22.0
3	4	1	1	7	1	1	23.2	26.1	29.5	23.9
4	3	1	0	4	0	3	29.9	30.5	28.7	29.3
5	2	3	1	2	1	1	30.1	30.6	31.2	30.5
6	0	1	1	1	0	0	27.6	30.8	28.2	31.2
7	3	4	2	2	1	0	not measured		29.0	27.8
8	2	9	1		1	0			32.5	28.3
9	0	5	10		0	1			29.3	30.6
10	0	3	7		0	2			26.1	29.3
11	0	6	3		1	0			25.2	23.2
12	0	1	1		0	0			26.6	27.7
13	0	1	10		1	2			25.4	24.5
14	1		2		1	1			27.3	26.4
15			1		1	2				
16			0		4	1	Avg per mouse/day			
17			0		1	1	23.0	26.6	26.8	25.6
18			0		1	5				
19			0		1	3				
20			1		6	1				
21			0		3	1				
22			0		1	1				
23			0		0	2				
24 to 30			0		0	0				
Survivors (30 days)	0	0	1	0	3	1				
Mean survival time (days)	2.86	5.86	9.04	3.18	16.03	14.20				
Median survival time (days)	2.0	7.0	9.1	2.9	17.0	16.0				
Standard error of mean	3.07	3.78	5.19	1.7	7.46	6.90				
Standard error of median	0.56	0.54	0.74	0.38	1.36	1.26				
	0.70	0.68	0.93	0.49	1.70	1.58				

\*Series A is a summary of 3 experiments started on 12/13 and 14/39 and 1/5/40. The mice received 2 billion, 175 million and 2 billion cocci on the respective dates. Therapy maintained 7 days after infection. Drug consumption was determined only in last experiment.

†Series B is a summary of 2 experiments started on 1/25 and 26/40. The mice received 2 billion and 6 billion cocci on the respective dates. Therapy maintained for 2 wks after infection. Drug consumption was determined in both experiments.



Treatment was carried out by administering the drug in the animals' diet. This method was selected because it is the simplest and at the same time, as McKee and her associates pointed out,<sup>6</sup> the surest means of maintaining a fairly even concentration of these drugs in the blood of the mice. The diet consisted of well ground Purina Dog Chow. The drug was added to this in an amount sufficient to give a 1% concentration and the two were mixed carefully and thoroughly. The mice were kept in individual cages. The drug-diet mixture, offered in containers as described by Bieter *et al*,<sup>7</sup> was weighed daily in order to ascertain the amount which had been consumed. Treatment was carried out in this way for 2 days prior to infection and, in the first series of experiments, for one week thereafter, in the second for 2 weeks.

**Results** The results are shown in Table I. The mice consumed approximately equal quantities of the 3 drug-diets. In the first series of experiments the mean survival times were 2.86 days for the untreated animals, 5.86 days for those treated with sulfapyridine and 9.04 days for those treated with sulfathiazole. These differences are statistically significant. When the median survival times are calculated the difference between the survival times of the sulfathiazole and sulfapyridine-treated animals (9 and 7 days respectively) is less obviously significant. Sulfathiazole and sulfamethylthiazole, as shown in the second series of experiments, were almost equally effective in prolonging the lives of the infected animals. The difference in the results with sulfathiazole in the two experiments is, of course, due to the fact that therapy was maintained twice as long in the second series. It is interesting that in both series the average mouse treated with sulfathiazole survived just 2 days after treatment was discontinued.

**Discussion** These results are in accord, as far as the comparison of sulfathiazole and sulfapyridine is concerned, with those of Barlow and Homburger<sup>8</sup> when allowance is made for the differences in the two techniques. They do not agree with these authors' results with sulfathiazole and sulfamethylthiazole since they found sulfamethylthiazole to be the more effective of the two compounds, and we found no therapeutic difference between them. The superiority of sulfamethylthiazole in the hands of Barlow and Homburger is probably

<sup>6</sup> McKee, C. M., Rake, Geoffrey, Greep, R. O., and van Dyke, H. B., *Proc. Soc. Exp. Biol. and Med.*, 1939, **42**, 417.

<sup>7</sup> Bieter, R. N., Larson, W. P., Cranston, E. M., and Levine, Milton J., *Pharm. and Exp. Therap. Proc.*, 1939, **65**, 3.

<sup>8</sup> Barlow, O. W., and Homburger, E., *Proc. Soc. Exp. Biol. and Med.* 1939, **42**, 792.

attributable to the fact that after the first 32 hours in their experiments the drugs were given in single daily doses and as sulfathiazole is excreted more rapidly than its methyl derivative, the blood levels obtained with it would have a lower daily average

*Summary and Conclusions* Sulfathiazole and sulfamethylthiazole, administered as 1% of the diet showed a distinct and equal therapeutic value in prolonging the lives of mice heavily infected with *Staphylococcus aureus*. Sulfathiazole proved to be somewhat more efficient in this respect than sulfapyridine

We are indebted to E. R. Squibb and Sons and the Calco Chemical Division of the American Cyanamid Company for the sulfathiazole used in these experiments and to the Department of Medical Research of the Winthrop Chemical Company for the sulfamethylthiazole

### 11311 P

#### Effect of Pregnenolone (17-Ethynyl Testosterone) on Genital Tract of Immature Female Rats

U. J. SALMON AND A. A. SALMON

*From the Laboratories of the Mount Sinai Hospital, New York City*

It has previously been shown that testosterone, when administered to immature female rats, exhibits 3 biological properties: (a) it is gynecogenic, causing premature opening of the vagina<sup>1</sup> and growth of the epithelial and muscular elements in both the vagina and uterus;<sup>2, 3</sup> (b) it is androgenic, causing growth of the clitoris and preputial glands;<sup>2, 3</sup> (c) it is hypophyseotropic, stimulating the hypophysis to secrete gonadotropic hormone which is manifested by growth of follicles and appearance of corpora lutea in the ovaries.<sup>2-7</sup>

<sup>1</sup> Batenand, A., and Kudzus, H., *Hoppe Seyler's Z.*, 1935, **73**, 237

<sup>2</sup> Korenchensky, V., Dennison, M., and Hall, K., *Biochem. J.* 1937, **31**, 780

<sup>3</sup> Salmon, U. J., *Endocrinology*, 1938, **23**, 779

<sup>4</sup> Salmon, U. J., *Proc. Soc. Exp. Biol. and Med.*, 1938, **38**, 352

<sup>5</sup> Nathanson, I. T., Franseen, C. C., and Sweeney, A. R., Jr., *Proc. Soc. Exp. Biol. and Med.* 1938, **30**, 385

<sup>6</sup> Starker, W. F., and Leatham, J. H., *Proc. Soc. Exp. Biol. and Med.*, 1938, **30**, 218

<sup>7</sup> Freed, S. C., Greenhill, J. P., and Soskin, S., *Proc. Soc. Exp. Biol. and Med.* 1938, **30**, 440

Recently, Inhoffen and coworkers<sup>8</sup> have added an ethinyl group to testosterone at the 17th carbon atom, producing a compound ( $\Delta^4$  pregnen-in-20-on-3-ol-17, 17-ethinyl testosterone, pregneniolone) which, chemically, is closely related to both testosterone and progesterone. Inhoffen and Holweg<sup>9</sup> have shown that this compound has a progesterone-like action in immature rabbits and is, furthermore, active when administered orally. Ruzicka, Hofmann and Meldahl<sup>10</sup> made essentially similar observations. In humans, the compound has been shown to produce a progesterone-like effect on the estrogen-primed endometrium<sup>11, 12</sup> and to cause uterine bleeding in cyclical and amenorrhoeic women.<sup>13</sup>

The present study was undertaken to determine the biological properties of this compound in the immature, intact and ovariectomized female rat. The pregneniolone\* was dissolved in sesame oil, in concentration of 1 mg per cc of oil, and administered subcutaneously. Twelve littermate immature, female rats (3 litters), 30 days of age, were injected daily with 0.5 mg of pregneniolone, for 3 days and sacrificed at the end of 96 hours.

Opening of the vagina occurred on the 3rd day, in all injected animals, after the animals had received a total of 1.5 mg of pregneniolone. Vaginal smears revealed a complete cornification reaction in all. In the animals sacrificed at the end of 96 hours, the ovaries did not reveal macroscopic evidence of follicle stimulation or corpora lutea, the uteri, clitoris and preputial glands, in each case, were definitely enlarged and abundant secretion could be expressed from the latter.

Microscopic examination of serial sections of the ovaries failed to reveal any evidence of follicle stimulation or corpora lutea. On section, the muscular coats of the uteri were found to be moderately hypertrophied, as compared with the controls. Microscopic examination of the vaginae revealed complete cornification of the mucosa and hypertrophy of the muscular coats.

<sup>8</sup> Inhoffen, H. H., Longemann, W., and Serini, A., *Ber. Deutsch. chem. Ges.*, 1938, **71**, 1024.

<sup>9</sup> Inhoffen, H. H., and Hohlweg, W., *Naturwissenschaften*, 1938, **26**, 96.

<sup>10</sup> Ruzicka, L., Hofmann, K., and Meldahl, H. F., *Helv. chem. Acta*, 1938, **21**, 372.

<sup>11</sup> Clauberg, C., and Ustün, Z., *Zentralbl. f. Gynäkologie*, 1938, **62**, 1745.

<sup>12</sup> Salmon, U. J., Walter, R. I., and Geist, S. H., *Proc. Soc. Exp. Biol. and Med.*, 1939, **40**, 252.

<sup>13</sup> Zondek, B., and Rozin, S., *Lancet*, 1939, **1**, 504.

\* For the pregneniolone used in this study, we are indebted to Dr. E. Schwenk, of the Schering Corporation, Bloomfield, N. J.

*Summary and Conclusions* It appears from this study that  $\Delta^4$  pregnen-in-20-on-3-ol-17 exhibits bisexual properties when administered subcutaneously in oil to immature, female rats, having an estrogen-like action on the uterus and vagina and an androgen-like action on the preputial glands and clitoris. The trophic effect on the genital tract appears to be a direct one and not mediated through the ovaries, as shown by the elicitation of similar effects in ovariectomized immature animals. Unlike testosterone, however, pregneninolone appears not to have a stimulating effect upon the gonadotropic activity of the hypophysis, as indicated by the absence of corpora lutea formation or evidence of follicle stimulation in the ovaries.

It is interesting to note that this compound possesses a unique variety of biological properties. In addition to its progesterone-like action on the endometrium, Courrier and Jost<sup>14</sup> have found that pregneninolone will maintain pregnancy in spayed rabbits and is androgenic in castrated rats and chicks. Furthermore, Emmens and Parkes<sup>15</sup> have shown that this compound has estrogen-like properties when administered to adult, female rats, as well as androgenic activity, as indicated by the capon comb growth. It is worthy of note that the introduction of the ethinyl group at the 17th carbon atom of testosterone produced a compound possessing properties that are characteristic of estrogens, androgens and progesterone and at the same time has resulted in a loss of hypophyseotropic potency—the power to stimulate gonadotropic hormone secretion by the hypophysis.

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<sup>14</sup> Courrier, R., and Jost, A., *C R Soc Biol Paris*, 1939, **130**, 1162.

<sup>15</sup> Emmens, C. W., and Parkes, A. S., *Nature*, 1939, **143**, 1064.

## Local Tissue Reaction to the Implantation of Crystals and Pellets of Estrogenic Hormone

S H GEIST, R I WALTER AND U J SALMON

*From the Laboratories of the Mount Sinai Hospital, New York City*

Deanesley and Parkes,<sup>1, 2</sup> in a study of the various factors which influence the rate of absorption of sex hormones in animals, demonstrated that the subcutaneous implantation of the pure dry hormone was the most efficient mode of administration. In studies in the human, we have shown<sup>3</sup> that, by means of the subcutaneous implantation of crystals of  $\alpha$ -estradiol benzoate it was possible to prolong the period of effectiveness of the hormone. This was demonstrated by (a) the long period of clinical improvement, (b) the persistence of estrogenic effects on the vaginal smear and (c) the prolonged pituitary inhibition in menopausal patients.<sup>4</sup> Subsequently, in an attempt to obtain more prolonged effects with the estrogenic substances, pellets of  $\alpha$ -estradiol and  $\alpha$ -estradiol benzoate were implanted subcutaneously.

One of the questions which immediately arose following the use of this procedure was, what effect the crystalline estrogens would have upon the contiguous subcutaneous tissues. It is our purpose, in this communication, to describe the local tissue reaction to the implanted estrogen crystals\* and pellets\*.

We have employed the implantation method in a series of 110 cases and from this series 14 patients (of whom 8 were spontaneous menopause, 4 surgical and 2 X-ray castrates), were selected for excision of the implanted hormone and surrounding tissues. Ten patients had been implanted with pellets (5  $\alpha$ -estradiol and 5  $\alpha$ -estradiol benzoate). All of these cases, before the implantation, had typical menopause symptoms and morphologic evidence of estrogen deficiency, as indicated by negative vaginal smears and biopsies of the vaginal mucosa and endometrium. The amount of hormone implanted varied from 15 to 50 mg. The individual pellets weighed

<sup>1</sup> Deanesley, R., and Parkes, A. S., *Proc Roy Soc B*, 1937, **124**, 279.

<sup>2</sup> Deanesley, R., and Parkes, A. S., *Lancet*, 1938, **2**, 606.

<sup>3</sup> Salmon, U. J., Walter, R. I., and Geist, S. H., *Science*, 1939, **90**, 162.

<sup>4</sup> Salmon, U. J., Geist, S. H., and Walter, R. I. *Proc Soc Exp Biol and Med*, 1940, **43**, 424.

\* For the crystals and pellets of  $\alpha$ -estradiol and  $\alpha$ -estradiol benzoate used in this investigation, we are indebted to Dr. E. Schwenk of the Schering Corporation, Bloomfield, N. J.

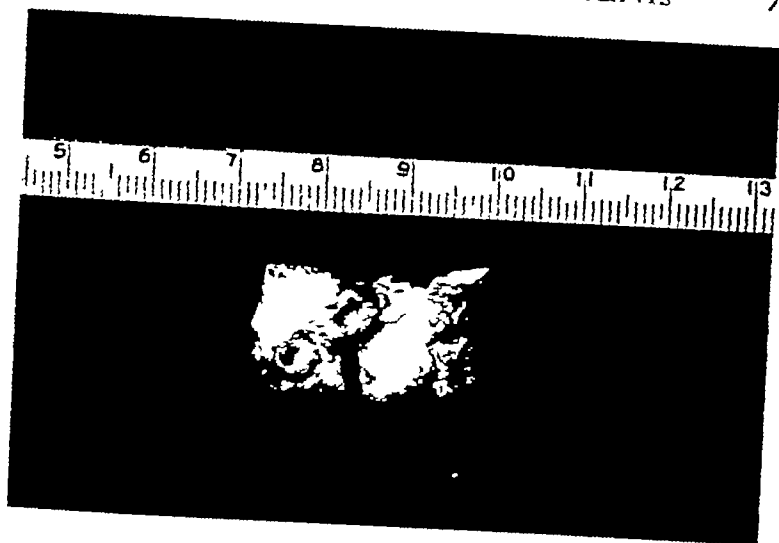


FIG 1

Case D.N. Natural size photograph of implantation site excised 88 days after implantation of 2 pellets of  $\alpha$ -estradiol benzoate showing capsule formation



FIG 2

Case D.N. Cross section through capsules ( $\times 10$ )

15 to 25 mg. Four patients were implanted with crystals of  $\alpha$ -estradiol and  $\alpha$ -estradiol benzoate, varying in weight from 10 to 23 mg. The implantation sites were excised under local (1% novocaine) anesthesia, at periods varying from 23 to 238 days after the implantation. An ample elliptical incision was made over the site of implantation in order to include the skin and surrounding tissues. The pellets were removed and the excised tissue was fixed in 10% formalin. Serial sections were made and stained with hematoxylin and eosin. In none of the cases implanted with crystals were we able to find any gross evidence of the crystals at the time of excision.

It was noted that a fairly uniform tissue reaction occurred in each case. The pellets were completely surrounded by a fibrous capsule which varied in thickness depending upon the duration of implantation. The pellets retained their original shape and consistency and, although intimately adherent to the capsule, could be easily shelled out. The subcutaneous tissues showed a typical foreign body reac-

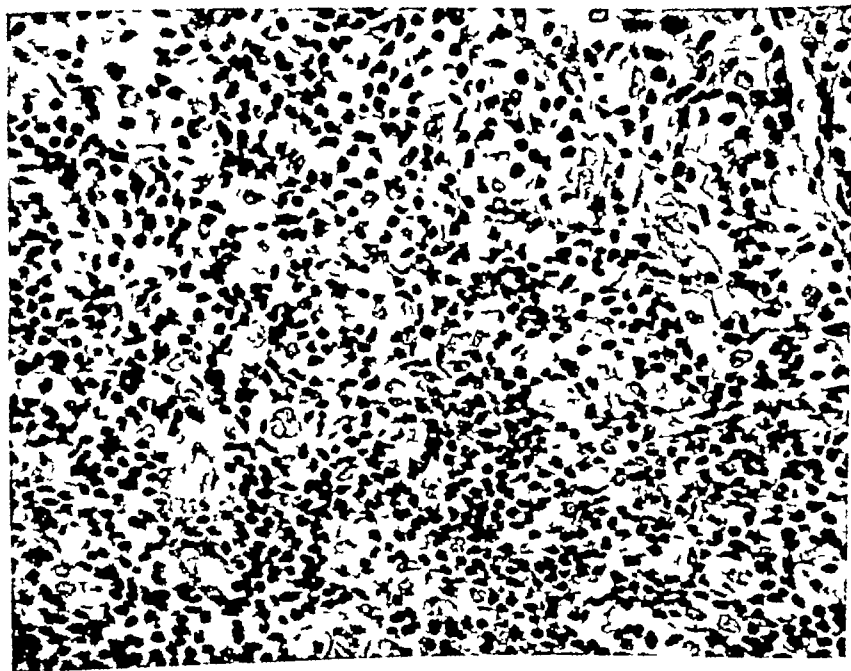


FIG. 3

Case D V. Section through middle layer of capsule showing round cells and giant cells ( $\times 400$ )

tion Each of the capsules showed essentially the same histologic picture The protocol of a typical case is presented here

This patient (D N ) was implanted in the subcutaneous tissue of the right thigh with 2 pellets of *a*-estradiol benzoate (total weight 50 mg) and the implant-site was removed 88 days later Fig 1 is a natural size photograph demonstrating the gross appearance of 2 capsules after removal of both pellets Fig 2 is a low power magnification of a cross section of the capsules illustrated in Fig 1 showing the sharply demarcated character and thickness of the capsules The significance of the thickness of the capsules will be discussed later

The capsule consists of 3 more or less distinct layers surrounding a central cavity The inner layer adjacent to the cavity consists of 2 zones (a) an inner zone which is composed of 1 to 3 layers of cells which are elongated narrow and eosinophilic and contain irregular nuclei which have no uniform cell position and (b) an hyalinized connective tissue zone containing scattered lymphocytes leucocytes



FIG 4.

Case L S Excised tissue 236 days after the implantation of 10 mg of *a*-estradiol crystals ( $\times 20$ ) Note tubercle like nodules



and some large pale-staining cells with oval nuclei. The nuclei of these large cells have a well-defined chromatin content, the cells resembling phagocytes. The middle layer (Fig 3) is a slightly wider area which is supported by an hyalinized connective tissue substratum and is infiltrated by many closely packed, small, round cells (lymphocytes and plasma cells), fibroblasts, occasional leucocytes, and a scattering of giant cells. The outer layer consists of a relatively narrow band of hyalinized fibrous tissue which contains some dilated lymphatic vessels. The surrounding fat tissue shows slight edema and contains a few lymphocytes and giant cells. The skin overlying the implanted pellets is normal.

On excision of the crystalline implantation sites, no macroscopic capsule could be seen. On cut section one could, however, discern multiple, pin-point grayish-white areas distributed throughout the subcutaneous fat tissue.

Fig 4 is a low power photograph of a cross section of an im-



Fig 5

Case L S Higher magnification ( $\times 100$ ) of single nodule indicated by arrow in Fig 4

plantation site removed from patient L S who had been implanted 236 days previously with 10 mg of  $\alpha$ -estradiol crystals. One can clearly see in this section at least 14 minute discrete, conglomerations of cells, which have the appearance of small tubercles. With higher magnification (Fig 5) the resemblance of these accumulations of cells to foreign body tubercles becomes even closer.

These tubercles consist of a central core of tissue containing several cavities of variable size, which are delimited by fine connective tissue septa. The latter contain many irregular nuclei which vary considerably in size and shape. Some of the nuclei are giant sized and no separate cell margins can be identified. This central core is surrounded by a zone of hyalinized tissue which contains numerous irregularly shaped cells with large vacuoles and a scattering of foreign body giant cells (Fig 6).

Surrounding the above described central structure are 3 layers of cells which resemble, in miniature, the 3 layers of cells which make up the gross capsule previously described.

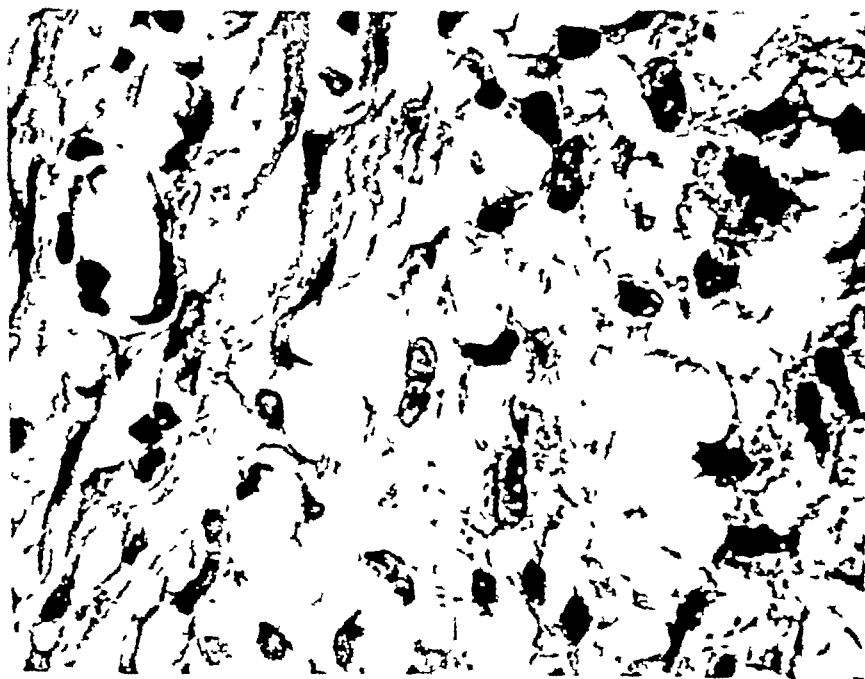


FIG 6

Case L.S. High power magnification ( $\times 1200$ ) of boxed in area of Fig 5 showing large multi nucleated vacuolated cells

*Summary and Conclusions*

The local tissue response to implanted crystals and pellets is a typical non-specific foreign body reaction. Following the implantation of crystals of  $\alpha$ -estradiol and  $\alpha$ -estradiol benzoate, the tissue surrounding the crystals responds by the formation of tiny nodules. In contrast to this type of reaction, a thick avascular capsule forms about the pellets of the implanted hormone. It is important to note that the epidermis overlying the implants showed no evidence of cellular atypism and, similarly, in no instance did the subcutaneous tissues in contact with and adjacent to, the implanted hormone show any abnormal cellular proliferation.

A significant observation which emerges from this study is the probable effect that the thick avascular capsule has on the absorption rate of the estrogenic substances employed. It was noted that the crystals had a strikingly more prolonged therapeutic and physiologic effect than the pellets composed of the same estrogenic substance and of comparable weight. It would appear therefore, that the capsule might have a marked retarding effect on the rate of absorption of the hormone. Apparently, with the passage of time, the absorption rate is progressively diminished by the growing thickness of the capsule around the pellet. After a period of approximately 3 months absorption is either completely stopped or so reduced as to have no demonstrable physiologic or therapeutic effect. It seems therefore that for purposes of implantation, pellets of  $\alpha$ -estradiol and  $\alpha$ -estradiol benzoate are not as efficient as crystals of the same chemical constitution.

## 11313 P

**Portals of Entry of Poliomyelitis Virus in the Chimpanzee \***

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The lack of any direct evidence concerning the portal of entry of poliomyelitis virus in man has led to many animal experiments. The use of the resistant rhesus monkey and of "monkey strains" of virus in this problem, however, is open to the objection that the experimental conditions at the outset are widely divergent from those under which the disease takes place in human beings. A closer approxima-

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\* Supported by a grant from the Commonwealth Fund

tion to the actual situation of the epidemic may be obtained by the use of human stools as a source of virus and the chimpanzee as a near substitute for man. The chimpanzee was selected for this purpose because of its phylogenetic relationship to man and because of a report by Müller<sup>1</sup> that during an outbreak of poliomyelitis in Cologne, Germany, 2 chimpanzees in a children's zoo apparently contracted the disease by natural contagion. One of these animals subsequently died and an examination of the central nervous system by Spielmeyer revealed lesions characteristic of poliomyelitis.

Five chimpanzees were inoculated with a pool of 7 different untreated human stools each of which had previously been found positive by intranasal inoculation into rhesus monkeys.<sup>2</sup> The results are briefly summarized in Table I. Three animals received large inoculations on 5 successive days by stomach tube, while the other two were given 2 cc of untreated stool intranasally and by mouth, respectively, on 3 successive days. In each instance special care was taken to exclude portals other than the one inoculated, and in the case of the last animals (A105-A106), both olfactory tracts had been sectioned intracranially 4 and 5 days previously. All animals except the first (A48, which received stool by stomach tube) contracted typical flaccid paralysis of the left arm. The animal with cut olfactory tracts which received stool by mouth had an initial right facial paralysis which later extended also to the left arm. In all, sections of the cervical cord showed heavy poliomyelitic lesions. Cord and stools from A83 and A105 reproduced the disease in rhesus monkeys. Despite the typical form of the paralysis and lesions in A71 virus could not be demonstrated in either the cord or stools. The stools and cord of A106 have not been tested for virus as yet.

The olfactory bulbs of A83 (inoculated intranasally) showed maximal perivascular cuffing, neuronophagia and cellular infiltration. In contrast to this the olfactory bulbs of A71 the normal animal receiving stool by stomach tube, showed no signs of virus invasion. There was evidence of a meningitis around the olfactory nerve fila which was in keeping with the generalized meningitis over all exposed pial surfaces of the brain. The bulbs of the animal subjected to bilateral olfactory tract section (A105) showed similar changes. There was a generalized meningitis (the spinal fluid white cell count was 900 per cu mm). There were also slight infiltrations of lympho-

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<sup>1</sup> Müller, W., *Monatsschr f. Kinderheilk.* 1935 **63**, 134.

<sup>2</sup> Howe, Howard A. and Bodin, David J. *Infect Dis.* 1940 in press.

TABLE I

Chimpanzee	Mode of inoculation (untreated stool pool)	Intranasal rhesus controls	Incubation period from day of 1st inoculation	Lesions		
				Olf bulbs	Spinal cord	Cord passage
A48, ca 5 yrs	Stomach tube, 95 cc stool pool	Prostrate	No paralysis			
A71, " 4½ "	Stomach tube, 250 cc stool pool	"	14 days, lt arm paralysed	Neg	++	Neg
A83, " 6 7 "	Intranasal, 6 cc stool pool	"	8 days, lt arm paralysed	++	++	Pos
A105, " 3½ "	Olf tracts cut, stomach tube, 235 cc stool pool	"	9 days, lt arm paralysed	Neg	++	Pos
A106, " 3½ "	Olf tracts cut, 6 cc stool pool by mouth	"	15 days, rt faec, lt arm paralysed	Neg	++	

cytes and losses of mitral cells which were probably the result of retrograde changes following the tract sections. The olfactory bulbs of A106 showed no inflammatory infiltrations. The completeness of the olfactory tract section in these cases rules out any possibility of virus having reached the brain by this route<sup>3</sup>†

The failure of chimpanzee A48 to contract poliomyelitis cannot readily be explained, since 2 intranasal rhesus controls were paralyzed in 10 days. A period of 10 days elapsed between the first and second stomach tube inoculations of 25 cc of stool. This was a relatively small dose as compared with those given by the same method to the other animals. On the sixth day following the first inoculation the animal's temperature rose from 100.4° to 104.6° but on the next day had dropped to its former level. The animal has remained entirely well for 6 months. Whether this fever indicated an abortive attack of poliomyelitis cannot be stated. In contrast with this is the finding that A106, a younger animal, became paralyzed after receiving only 6 cc of stool by mouth. The fact that the initial paralysis was bulbar suggests the oral cavity and pharynx as a portal of lower threshold than the gastrointestinal tract.

*Summary* Typical poliomyelitis has been produced in 4 chimpanzees by the intranasal, intragastric and oral inoculation of untreated human stools. In 2 instances the olfactory portal was ruled out by section of both olfactory tracts.

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<sup>3</sup> Brodie, M., and Elvidge, A. R., *Science*, 1934, **79**, 235, Schultz, E. W., and Gebhardt, L. P., *Proc. Soc. Exp. Biol. and Med.*, 1934, **31**, 728, Howe, Howard A., and Ecker, Robert S., *Proc. Soc. Exp. Biol. and Med.*, 1937, **37**, 125.

†Vignec, Paul and Trask's<sup>4</sup> feeding experiments on monkeys unfortunately do not exclude this possibility.

<sup>4</sup> Vignec, A. J., Paul, J. R., and Trask, J. D., *Proc. Soc. Exp. Biol. and Med.*, 1939, **41**, 246.

# Substance in Kidneys and Muscle Eliciting Prolonged Reduction of Blood Pressure in Human and Experimental Hypertension \*

I H PAGE, O M HELMER, K. G KOHLSTAEDT, P J FOUTS, G F KEMPf AND A C CORCORAN

From the Lilly Laboratory for Clinical Research, Indianapolis City Hospital, Indianapolis, Indiana

Evidence published elsewhere<sup>1-4</sup> strongly suggests that an inhibitor to the pressor action of angiotonin and renin is contained in kidneys and probably muscle and lung

**Methods** Extracts prepared from fresh kidneys, lung and muscle were tested for their ability to reduce arterial pressure in dogs made hypertensive by the silk or cellophane perinephritis method.<sup>5</sup> Blood pressure was determined by direct femoral arterial puncture. Hypertensive rats (Drury method<sup>6</sup>) were also employed, the blood pressure being measured in the tail by the method of Williams, Harrison and Grollman.<sup>10</sup> The blood pressure of the animals was stabilized at the elevated pressure before extracts were assayed. Some of the dogs exhibited the syndrome of malignant hypertension. Extracts were given by injection subcutaneously, into muscles or by mouth.

The extracts have been tested also on patients with typical essential hypertension and malignant hypertension after the patients had been studied for long periods in the Lilly Clinic. The studies were conducted much as those described previously in evaluation of the various surgical treatments of hypertension (Page and Heuer<sup>11</sup>).

Several experiments have been performed to ascertain whether the

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\* A preliminary report of this work was made at the American Association for the Advancement of Science, Section N, Columbus Ohio December 27 1939

1 Page, I H, *J Exp Med*, 1939, **70**, 521

2 Kohlstaedt, K. G, Helmer, O M, and Page, I H, *Proc Soc Exp Biol. AND MED*, 1938, **30**, 214

3 Kohlstaedt, K. G, Page, I H, and Helmer O M *Am Heart J*, 1940, **10**, 92

4 Page, I H, and Helmer, O M, *J Exp Med* 1940, **71**, 29

5 Tigerstedt, R, and Bergmann, P G, *Skand Arch Physiol*, 1898, **8**, 223

6 Page, I. H, and Helmer, O M, *J Exp Med* 1940, **71**, 495

7 Goldblatt, H, Lynch, J, Hanzal, R F, and Summerville, W W, *J Exp Med*, 1934, **50**, 347

8 Page, I H., *J A M A*, 1939, **113**, 2046

9 Drury, D R, *J Exp Med.*, 1938, **68**, 693

10 Williams, J R, Jr, Harrison, T R, and Grollman, A, *J Clin Invest*, 1939, **18**, 373

11 Page, I. H., and Heuer, G J, *Arch Int Med*, 1937, **59**, 245

ability of plasma to activate renin has been reduced after administration of extracts. The method employed was that of Page and Helmer<sup>6</sup>

*Extracts of Kidney and Muscle* Extracts of minced tissues were prepared from pork kidney and from rat, rabbit and beef muscle by 6 general methods of extraction, (a) with saline, (b) with acetone, (c) with alcohol, (d) by fractional precipitation with  $(\text{NH}_4)_2\text{SO}_4$ , (e) by isoelectric precipitation of water and saline extracts, and (f) saline and acetic acid followed by heating to  $58^\circ \text{C}$ . The materials obtained were concentrated *in vacuo* at low temperatures, and, in most cases, dialyzed for 24 hours or more. The concentrated solutions were passed through Seitz filters before intramuscular administration to human beings. Details of the procedures used and results obtained are shown in the accompanying tables.

*Relative Potency of the Various Extracts in Dogs and Rats* The filtrate from the isoelectric precipitation of saline extracts of kidney seemed most effective. The amount of extract equivalent to 300 g of original kidney when injected intramuscularly in dogs over a period of 2 to 4 days, caused marked reduction in arterial pressure (Figs 1 and 2). The isoelectric precipitate also contains some of the inhibitor.

TABLE I.

Effect of Tissue Extracts on the Arterial Blood Pressure of Dogs with Hypertension.

Type of extract	Estimated* effectiveness	G of fresh kidney required to produce maximum fall in B.P. from hypertensive ( $>180 \text{ mm Hg}$ ) levels
Saline extract of whole kidney	+++	600
Isoelectric precipitate of saline kidney extract	+++	400
Filtrate of an isoelectric precipitate of a saline kidney extract	++++	300
0.3 saturated $(\text{NH}_4)_2\text{SO}_4$ precipitate of saline kidney extract	++	—
0.3 to 0.8 saturated $(\text{NH}_4)_2\text{SO}_4$ precipitate of kidney extract	0	—
70% acetone extract of kidney	+++	2100
50 to 70% alcohol extract of kidney	++	6000
Extracts of lung	+	—
Filtrate of heated saline-acetic acid extract	++++	960

\*Results given in table are an estimate of the effectiveness of the extracts in reducing blood pressure based on the following grades

- + Transient effect
- ++ Some reduction in blood pressure but not reduced to normal level.
- +++ Definite reduction in blood pressure but not sustained
- ++++ Marked reduction (reduced to normal level), well sustained



# Substance in Kidneys and Muscle Eliciting Prolonged Reduction of Blood Pressure in Human and Experimental Hypertension \*

I H PAGE, O M HELMER, K G KOHLSTAEDT, P J FOUTS, G F KEMPF AND A C CORCORAN

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Evidence published elsewhere<sup>1-5</sup> strongly suggests that an inhibitor to the pressor action of angiotonin and renin is contained in kidneys and probably muscle and lung

**Methods** Extracts prepared from fresh kidneys, lung and muscle were tested for their ability to reduce arterial pressure in dogs made hypertensive by the silk or cellophane perinephritis method.<sup>6</sup> Blood pressure was determined by direct femoral arterial puncture. Hypertensive rats (Drury method<sup>9</sup>) were also employed, the blood pressure being measured in the tail by the method of Williams, Harrison and Grollman.<sup>10</sup> The blood pressure of the animals was stabilized at the elevated pressure before extracts were assayed. Some of the dogs exhibited the syndrome of malignant hypertension. Extracts were given by injection subcutaneously, into muscles or by mouth.

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11 Page, I H, and Heuer, G J, *Arch Int Med*, 1937, **50**, 245

when injected intramuscularly. The alcohol extracts were the least effective.

It will be noted from Table II that 70% acetone extracts of muscle, 50% alcohol extracts, and the isoelectric filtrates and redissolved precipitates at pH 4.2 are most active. Poor results were noted with 70% acetone extracts of rabbit muscle in which an average dose of

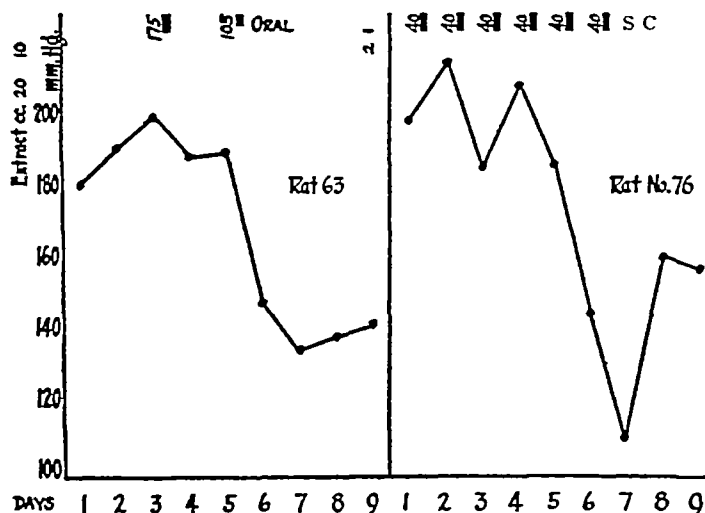


FIG 3a

Filtrate given orally after isoelectric precipitation equivalent to 380 grams of beef muscle

FIG 3b

Filtrate given subcutaneously Same extract as 3a

TABLE II.

Effect of Muscle Extracts on Arterial Blood Pressure of Rats with Hypertension

Type of extract	Estimated effectiveness	G of fresh muscle required—Avg
70% acetone	++++	162 (105*)
90% acetone precipitate from 70% extract	+++	190
90% " filtrate " " "	++	220
50% " extract of 70% precipitate	+	380
50% acetone extract	—	360
50% alcohol " "	++++	190
70% " " "	+++	520
50% alcohol in presence of 10% sodium chloride	—	250
0.3 saturated ammonium sulphate	++	—
0.3-0.5 saturated ammonium sulphate	+	—
0.5 full saturated ammonium sulphate	++	—
Isoelectric precipitate	+++	110
Filtrate of an isoelectric precipitate	++	149
Filtrate of heated saline acetic acid extract	++++	126

105\* Average dose of rat muscle extract 162 g includes both rat and rabbit.

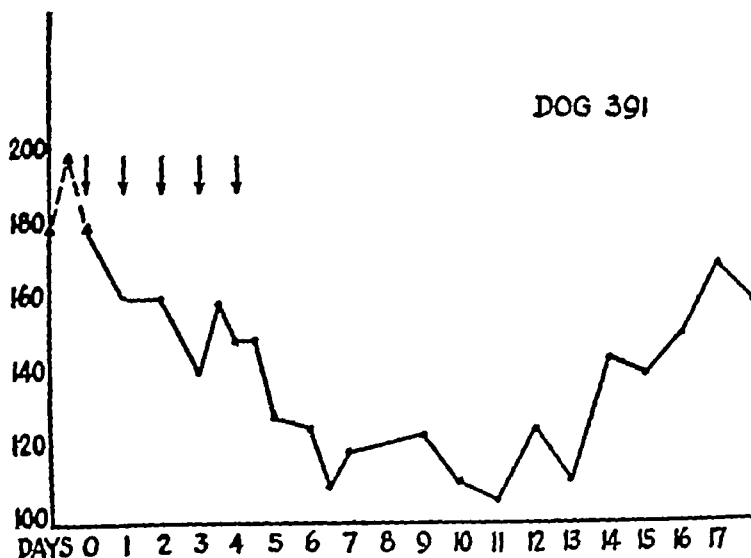


FIG 1

Aqueous extract equivalent to 120 grams of beef kidney given to a hypertensive dog

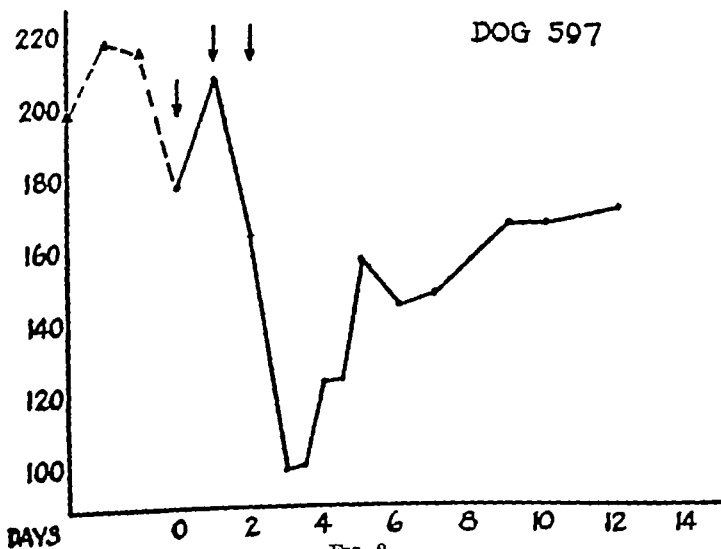


FIG 2

Filtrate after isoelectric precipitation equivalent to 100 grams of kidney daily

The acetone extracts of the kidneys were effective. In only one instance did a 70% acetone extract fail to reduce the dog's pressure

TABLE III  
Effect of Injection of Kidney Extracts on Activator Content of Hypertensive Dog's Blood  
Dog No. 101—Uninephrectomy plus colophane on remaining kidney

Date	Infusion drop rate per min	Substance perfused	Reduction of flow time in min	% reduction of flow	B.P. in mm Hg	Extract
11 20 30	110	2 plasma of dog No. 101	0	0	184	None
	111	2 " normal dog	0	0		
	110	2 normal dog plasma + reulu	1	30		
	115	2 No. 101 + reulu	1	68		
12 0	131	2 No. 101	0	0		
	135	2 normal plasma	0	0		
	112	1 No. 101 + reulu	1/2	20		
	105	1 normal plasma + reulu	3	12		
	112	2 angiotonin + 0.2 cc No. 101	1 1/2	31		
	130	2 " + 0.2 " normal plasma	1	61	112	Saline extract of kidney, concentrated
12 20	117	2 normal plasma	0	0		
	110	2 No. 101	0	0	170	None
	150	1 normal plasma + reulu	1 1/2	12		
	117	1 No. 101 + reulu	5 1/2	81		
	114	2 angiotonin + 0.2 cc normal plasma	1 1/2	20		
	118	2 " + 0.2 " No. 101	1 1/2	61		
1 20 10	95	1 normal plasma	0	0		
	91	1 No. 101	0	0	63	50% alcohol oral extract
	91	1 normal plasma + reulu	2	53		
	91	1 No. 101 + reulu	1	20		
	93	2 No. 101 + 0.2 cc angiotonin	1/2	12		
	93	2 normal plasma + 0.2 cc angiotonin	2	30		

250 g muscle had little effect, while 105 g of rat muscle seemed quite effective. The data at hand do not establish a similar difference between beef and rat muscle.

*Effect of Kidney and Muscle Extracts on Patients* Eight patients have been treated with extracts. In all a sharp prolonged fall in arterial pressure occurred whether the hypertension was of the malignant or essential variety. Symptomatic improvement has been marked. After discontinuing the extracts for 3 to 5 days the blood pressure tends to rise again. What we consider to be a typical curve is illustrated in Fig. 4.

*Effect of Kidney Extracts on Renin- and Angiotonin-Activator* Plasma from hypertensive dogs exhibits greater than normal ability to cause vasoconstriction when renin or angiotonin is added to it and the mixture perfused through an isolated rabbit's ear (Table III). When the arterial pressure is reduced by means of kidney extracts, this enhanced ability is reduced or lost.

*Discussion* There seems little possibility that the observed prolonged fall in arterial pressure could be due to any of known depressor substances for 3 reasons. First, the fall occurs very slowly—from 12 to 48 hours—depending on dosage. Second, all crystalloidal

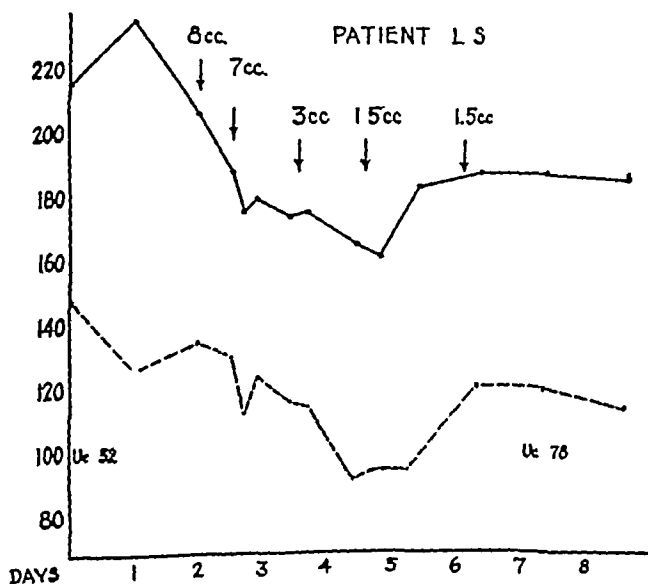


FIG. 4.

Administration subcutaneously of isoelectric filtrate equivalent to 800 grams of kidney to a patient with malignant hypertension. Urea clearance before extract was 52 percent of normal and 7 days later 78 percent of normal.

## 11315

**Experimental Hypoprothrombinemia with Anti-Prothrombin Serum**

L M TOCANTINS

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A transitory reduction in the plasma prothrombin of rabbits may be produced by injecting into these animals an antiserum prepared in the guinea pig against fibrinogen-free rabbit prothrombin

Fibrinogen-free prothrombin was isolated from the plasma of rabbits as follows<sup>1</sup> About 100 cc of blood was collected from a large rabbit by cardiac puncture, in 5 cc of 5% sodium citrate, the plasma was separated off, passed through a Berkefeld V filter and the clear filtrate then heated carefully to 54-56°C for 2 minutes, the precipitated fibrinogen being filtered off and discarded The plasma was then diluted 10 times with distilled water, and 1% acetic acid was added slowly with constant stirring until a pH of 6.0 was reached The precipitate that formed was, after centrifugation, diluted in about 5 cc of salt solution, to which a few drops of 0.5% sodium bicarbonate were added On standing at room temperature, most of the precipitate was dissolved To prepare the antiserum the prothrombin solution from 100 cc of blood was injected intraperitoneally into a guinea pig weighing no less than 600 g, 5 times, at approximately 6-day intervals, the animal being killed and bled out 10 days after the last injection Rabbits weighing between 2.5 and 3.0 kg were used, they were fed a mixture of clover hay, timothy, oats, corn and dog chow Blood was drawn from the heart or ear vein into a syringe containing 1.34% sodium oxalate solution (0.2 cc of the solution, 1.8 cc of blood) Prothrombin was measured according to the method of Quick<sup>2</sup> With each determination in the experimental animals, control determinations were performed on plasma from normal rabbits

Mixing increasing dilutions of the antiserum with a clear prothrombin solution brought about flocculation and precipitation of the prothrombin in dilutions up to 1:256 By incubating the undiluted antiserum with a given amount of oxalated rabbit plasma for 24 hours and following it with the addition of thromboplastin and calcium chloride it was possible to demonstrate a complete inacti-

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<sup>1</sup> Ferguson, J. H., *J. Lab. and Clin. Med.*, 1938, **24**, 273

<sup>2</sup> Quick, A. J., *Am. J. Physiol.*, 1937, **118**, 260

substances would have been lost during dialysis, for the inhibitor is non-dialyzable. Third, the reduction in pressure remains for several days in contradistinction to the transient reduction resulting from injection of histamine, choline, adenylic acid, etc.

Injection of kidney and muscle extracts reduce or abolish the ability of the plasma to activate renin and angiotonin. This may be interpreted as either reduction in the amount of activators or increase in the amount of inhibitors.

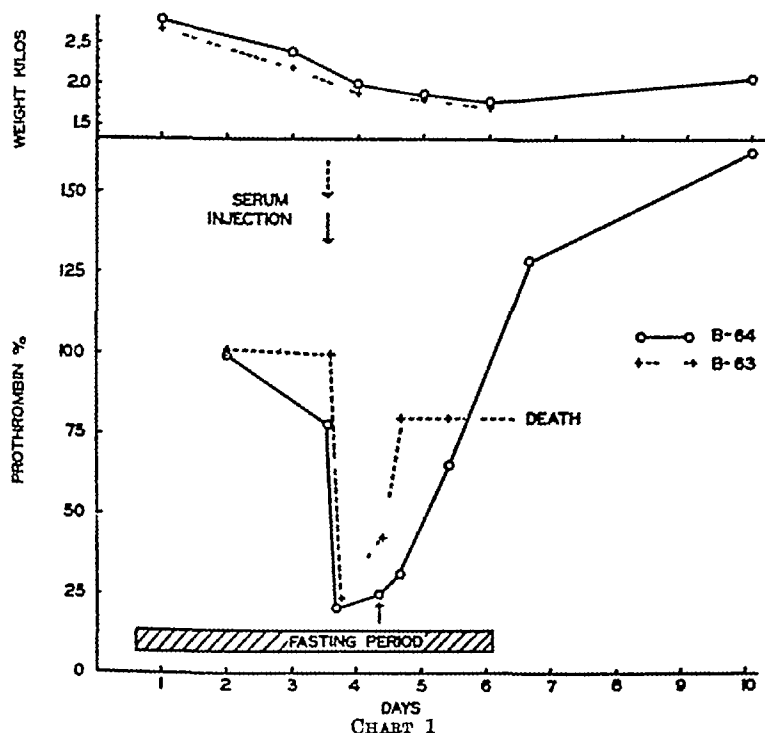
It has seemed of especial interest that in 4 dogs suffering from the syndrome of malignant hypertension that injection of extracts has reduced their blood pressure and caused improvement in the signs in the eyegrounds. Two human beings with malignant hypertension have also been treated. The fall in arterial pressure has been striking (Fig. 4). Urea clearance in these patients also exhibited a change for the better. It is too early to assert that over prolonged periods these extracts will have therapeutic value. Animal tests suggest that they may have value.

Most of our extracts have been given by injection, but the few that we have tried by mouth in rats have proven effective. Harrison, Grollman and Williams were the first to show that kidney extracts were effective by mouth.<sup>†</sup>

*Summary* Extracts of kidney and muscle effectively reduce arterial pressure in hypertensive human beings, dogs and rats for prolonged periods and simultaneously reduce the ability of plasma to activate renin and angiotonin.

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<sup>†</sup> The present results on extracts of kidneys, although arrived at by an entirely independent route of investigation, are in accordance with those of Harrison, Grollman and Williams which are now in press.

EFFECT OF INJECTION OF ANTIPROTHROMBIN SERUM  
IN FASTING RABBITS

Rabbit B 64 received 2 injections of the antiserum (solid arrow) and survived the experiment. Rabbit B 63 received 1 injection only (broken arrow) and died on the following day from a hemopericardium

ever observed to go below 20% of normal, this may explain the relative scarcity of hemorrhagic manifestations encountered

*Summary* An antiprothrombin serum may be prepared in the guinea pig by injecting it with fibrinogen-free prothrombin isolated from rabbit plasma. Intravenous injections of this antiserum in fasting rabbits produce a hypoprothrombinemia of a transient and moderate character



vation of the prothrombin in that plasma, and some reduction in prothrombin activity in dilutions of the antiserum up to 1-128

Subcutaneous injections into 2 rabbits in doses of 0.2 to 0.4 cc per kg body weight of the animal produced no detectable external changes either locally or generally. In this respect the serum differs from antiplatelet serum which produces large local hemorrhages when injected subcutaneously.<sup>3</sup> In the doses employed, injections of the antiserum intravenously in 4 healthy, nonfasting animals produced only a very transitory reduction in the prothrombin, varying between 25 to 40% of normal, detected usually between 15 and 20 minutes after an injection. There was a quick recovery by the end of the first hour and no subsequent reduction was detected. Repetition of the injection the next day brought about a similar response, followed later by a rise of the prothrombin concentration above normal and failure of subsequent injections to bring about a drop.

In 4 animals that had been fasting for 3 to 4 days it became easier to produce and maintain a hypoprothrombinemia by this method. Chart I illustrates the effects of one or more intravenous injections in 2 of these rabbits deprived of food for 3 days before the antiserum was given and while the experiments were being carried out. Fasting for 3 days in itself produces only a very slight reduction in the plasma prothrombin. Two animals died during the experiments from hemorrhage induced by cardiac punctures. When size of the veins made it possible all specimens in one animal were collected from the ear vein. Selection of animals with large ear veins makes it possible to collect rapidly the required amount of blood.

The rapidity with which the prothrombin returned to normal after being reduced by the injection of antiprothrombin serum is another indication of the great lability of this substance. The body reserves of vitamin K and of whatever other substances are required for the production of prothrombin by the liver seem to make a preliminary period of fasting necessary to demonstrate a clear-cut diminution of the prothrombin after injection of the antiserum.

The fact that some of the prothrombin in the plasma may be associated with other protein fractions besides the globulin may partially explain the moderate and transitory nature of the reduction in the plasma prothrombin; the antiserum may only destroy that portion of the prothrombin linked with the globulin fraction. Heating of the plasma to precipitate the fibrinogen may have also precipitated or destroyed some of the prothrombin and affected the antigenic properties of this protein. In no instance was the plasma prothrombin

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<sup>3</sup> Tocantins, L. M., *Arch. Path.*, 1936, **21**, 69

of age and 5 litters (26 animals) at the age of 87 days. Daily observations were made of the gross behavior of the animals and weekly weights were determined. At autopsy, the weight of the pituitary, gonads, adrenals, thyroid, spleen and kidneys was taken and compared with the body weight. The neck region was thoroughly examined at autopsy and all suspicious tissue subjected to microscopic examination of stained sections. All tissues were routinely examined microscopically using hemotoxylin and eosin stained celloidin sections.

Daily observation of the thymectomized and control animals maintained under the same experimental conditions (diet and handling) demonstrated no apparent difference in the gross behavior, size, time of opening of the eyes, eruption of the incisors and ears, descent of the testes, opening of the vagina, or character of the hair. Body weights and growth curves were of the same character. At autopsy, and after microscopic examination, the neck region of 2 of the 29 thymectomized animals was found to contain small amounts of thymic tissue (estimated at about 1/7th of the normal). Histological examination of the tissues removed at autopsy revealed no apparent difference between thymectomized and controls. There was no arrest of spermatogenesis in the testes of either age group as compared with the controls.

*Conclusions* It is felt that on the basis of the reports quoted and on the above data, thymectomy by surgical methods has no apparent effect on the growth, development, or endocrine organ differentiation of the rat when performed at the age of 1-2 days and studied past the onset of puberty. Since surgical thymectomy results in no apparent effects as studied above, it is probable that irradiation thymectomy can have no further effect, and that the cause of the effects described<sup>1, 2</sup> must be sought elsewhere.

## Effect of Thymectomy in Immature Rats \*

WILLIAM O REINHARDT (Introduced by Herbert M Evans)

*From the Department of Anatomy and the Institute of Experimental Biology,  
University of California, Berkeley, California*

It has been reported<sup>1 2</sup> that Roentgen irradiation of the thymus of 2-day-old rats results in the temporary arrest of development of the spermatogenic tissue of the testis. An analysis of recent literature shows that surgical thymectomy has been carried out on the following age groups of rats: female, age 1 day and male and female, age 21 days;<sup>3 4</sup> male and female, age 10-23 days;<sup>5</sup> male, age 25 days;<sup>6</sup> and male and female, age 21 days.<sup>7</sup> In none of these experiments were significant effects noted on growth, development, age of puberty, fertility, or differentiation of the endocrine organs.

With the above reports in mind and in order to explain if possible the difference between surgical and Roentgen ray thymectomy in immature animals, the following experiment was carried out.

Thymectomy was performed on half the animals of 10 litters of the Long-Evans strain of rats. Nine litters were operated upon at 4 days of age and one litter at 2 days of age. A total of 46 animals were employed of which 30 were males and 16 were females. The operated animals, both thymectomized and sham operations, were distributed according to littermate controls of equal body weight and size. Thymectomy was performed under ether anesthesia using a midline neck incision involving splitting of the upper part of the sternum, the thymus gland being readily exposed, and removed en masse by blunt dissection. Postoperative complications were negligible and the animals recovered from the effects of the operation without harm. Five litters (20 animals) were autopsied at 33 days.

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\* Aided by grants from the Board of Research of the University of California and the Rockefeller Foundation.

<sup>1</sup> Shay, H., Gershon Cohen, J., Fels, S., Meranze, D., and Meranze, T., *J Am Med Assn.*, 1939, **112**, 290

<sup>2</sup> Gershon Cohen, J., *et al*, *Science*, 1938, **87**, 20

<sup>3</sup> Andersen, D. H., *J Physiol*, 1932, **74**, 49

<sup>4</sup> Andersen, D. H., *J Physiol*, 1932, **74**, 207

<sup>5</sup> Pappenheimer, A. M., *J Exp Med.*, 1914, **19**, 319

<sup>6</sup> Hashimoto, E. I., and Freudenberger, C. B., *J Am Med Assn*, 1939, **112**,

1680

<sup>7</sup> Segaloff, A., and Nelson, W. O., *Anat Rec*, 1940, **76** (2), Supplement No 2, 50

animals lived 19 to 48 hours after the hypoglycemia was terminated

Animals in Group 3 were not studied as carefully as the above, but are added because they showed inability to maintain body temperature. They remained in a state of coma until death which occurred from 10 to 18 hours after the interruption of the hypoglycemia. Because of the poikilothermia it is assumed that the damage is at the midbrain level or lower.

Finding clinical evidence of damage at a certain level in the nervous system does not also establish the contention of simultaneous injury of the higher centers. This assumption appears warranted at this time from the clinical course of the animals under the experiment. During the induction of hypoglycemia, the gradient depression of level after level of nervous functions is easily followed and on termination of the hypoglycemia the reversal of this order is obtained. Histopathologic studies are in preparation to establish the proof for this contention.

The demonstration of a gradient effect by insulin in the central nervous system may have a very significant bearing on insulin shock treatment. The effects of this therapy are directed towards psychotic symptoms presumably arising in the highest cerebral centers. Therapeutic effects of the same type now obtained may occur with milder but more prolonged degrees of hypoglycemia, still capable of affecting the highest centers but sparing the lower medullary centers. This would remove the present hazards of shock therapy. This will be subjected to clinical verification.

The technic involved in this study may have an advantage over operative transection or the vascular decerebration of Pollack and Davis for studying the different physiologic strata of the central nervous system. It may be that with insulin the cleavage is along more definite physiological lines than is possible with these other "anatomic" methods.

Our cordial appreciation is expressed to Dr. Clinton H. Thienes of the department of pharmacology and to Dr. Douglas R. Drury of the department of physiology, for both facilities and many helpful suggestions.

## Decorticate and Decerebrate Preparations Produced by Insulin Shock.\*

EUGENE ZISKIND AND DAVID B TYLER (Introduced by C H Thienes )

*From the University of Southern California, School of Medicine, Los Angeles*

During insulin shock induced therapeutically there is a progressive depression of nervous functions, in a gradient manner, from the highest (cortical) levels to lower subcortical levels (striatum, mid-brain, medullary) which is temporary and reversible.<sup>1, 2</sup> It was decided to demonstrate this gradient effect clinically with the production of animal preparations showing permanent, irreversible lesions at progressively lower levels in the nervous system by prolonging the period of hypoglycemia

Cats, fasted 18 hours, were given 15 to 20 units of insulin per kilo body weight. When clinical signs indicated early medullary decompensation, small amounts of glucose were given intraperitoneally to prevent death and still maintain a marked degree of hypoglycemia. Hypoglycemia was terminated after 12 to 20 hours. Persistent brain damage was observed in 17 animals. Group 1 consisted of 5 animals with variable degrees of cortical damage. Group 2 totaled 8 animals with cortical plus subcortical injury ("thalamic preparations"?) Group 3 included 4 cats with loss of function at the midbrain level or lower.

The decorticate preparations (Group 1) showed impairment of vision, impairment of placing and hopping reactions (Bard), slight impairment of righting reactions, difficulty in feeding self, impairment of cleaning habits, restlessness and absence of rage reaction when confronted by a dog. They were able to walk and maintained normal body temperature. These preparations were kept alive from 6 to 90 days.

Preparations in Group 2 showed in addition to the above, inability to walk, decerebrate rigidity, increased tonic neck reflexes and mock rage. These animals were also able to maintain body temperature. These signs point to loss of function at the "thalamic level." The

\* The authors are indebted to Eli Lilly Company for the insulin utilized in this study.

1 Angyal, L. V., *Z f d ges Neurol u Psychiat* 1937, 157, 35

2 Frostig, J. P., *Arch Neurol and Psychiat*, 1938, 39, 219, also table in *Am J Psychiat*, 1939, 96, 373

animals lived 19 to 48 hours after the hypoglycemia was terminated

Animals in Group 3 were not studied as carefully as the above, but are added because they showed inability to maintain body temperature. They remained in a state of coma until death which occurred from 10 to 18 hours after the interruption of the hypoglycemia. Because of the poikilothermia it is assumed that the damage is at the midbrain level or lower.

Finding clinical evidence of damage at a certain level in the nervous system does not also establish the contention of simultaneous injury of the higher centers. This assumption appears warranted at this time from the clinical course of the animals under the experiment. During the induction of hypoglycemia, the gradient depression of level after level of nervous functions is easily followed and on termination of the hypoglycemia the reversal of this order is obtained. Histopathologic studies are in preparation to establish the proof for this contention.

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Our cordial appreciation is expressed to Dr. Clinton H. Thienes of the department of pharmacology and to Dr. Douglas R. Drury of the department of physiology, for both facilities and many helpful suggestions.

## Preparation and Properties of Soluble Bacterial Enzymes Which Decompose Creatinine

M J CARL ALLINSON (Introduced by H H Beard)

*From the Louisiana State University School of Medicine, New Orleans*

Several years ago, soil bacteria capable of producing adaptive enzymes which specifically decompose creatine and creatinine were isolated<sup>1, 2</sup> This report presents preliminary observations on the manner of preparation and properties of soluble enzymes prepared from one species (NC) of these bacteria

When the washed cells are desiccated by various methods a portion of the enzymic system which decomposes creatinine is rendered soluble This soluble portion can be obtained by adding water to the dried suspension, centrifuging, and collecting the clear supernatant solution The methods that have been used include grinding with abrasive materials, such as pumice-stone, desiccation with phosphorus pentoxide and sulphuric acid, and desiccation with alcohol-ether, or acetone-ether

The method of assay employs 0.5 mg of neutral creatinine-hydrochloride as substrate, 0.5 cc of 0.5 M phosphate buffer pH 7.0, the substance being tested, and water to 5 cc The suspension is incubated at 38° to 40°C for 30 minutes The undecomposed creatinine is determined colorimetrically after the addition of alkaline picrate

The activity of the preparation obtained under the above conditions represents about 10% of the activity of the original cells when assayed under the same conditions The properties of the extract differ in certain respects from the original cells Sodium cyanide  $10^{-3}$ M, and thymol inhibit the cells but not the extract Toluene and formaldehyde depress the activity of the cells and extract Sodium fluoride,  $10^{-3}$ M, does not inhibit the cells or extract

Studies on the purification of the enzymes are in progress

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<sup>1</sup> Miller, B F, and Dubos, R J, *Proc Soc. Exp Biol. and Med.*, 1936, **35**, 335

<sup>2</sup> Dubos, R J, and Miller, B F, *J Biol Chem*, 1937, **121**, 429

## 11319 P

## Effect of Testosterone Propionate Upon Thyroid and Parathyroid Glands of Intact Immature Female Rat.\*

IRA T NATHANSON, AUSTIN M BRUES AND RULON W RAWSON  
(Introduced by Joseph C Aub )*From the Laboratories of the Collis P Huntington Memorial Hospital, Harvard University*

In previous communications it was shown that testosterone propionate acts directly upon the vagina and uterus of the immature female rat<sup>1</sup>. It was also determined that it stimulates the ovary indirectly by way of the anterior hypophysis<sup>1</sup> even in the absence of the adrenals<sup>2</sup>. It is the purpose of this paper to record the effect of testosterone propionate upon the histological appearance of the thyroid and parathyroid gland of the intact immature female rat.

*Method of Experiment* Twenty-one immature rats of the Wistar strain weighing between 40 and 48 g and approximately 4½ weeks of age, were divided into 2 groups of 12 and 9 respectively. Five mg of testosterone propionate<sup>†</sup> in 0.2 cc of sesame oil were given in one dose to each of the first group (12) and 0.2 cc of sesame oil without the hormone were given to the second group (9) to serve as controls.

*Group I (Testosterone treated group)* Twelve hours after giving testosterone colchicine (0.05 mg per 50 g of body weight) was given subcutaneously to one animal. This animal was sacrificed 12 hours later (24 hours after testosterone). Thereafter one animal was sacrificed every 24 hours, one dose of colchicine having been given 12 hours previously.

*Group II (Controls)* Three animals each were sacrificed 72, 144 and 288 hours respectively after the onset of the experiment. Two of each group of 3 were given colchicine 12 hours before. The remaining animals received no other treatment.

\* This investigation was supported by a grant from the Committee on Research in Endocrinology, National Research Council.

<sup>1</sup> Nathanson, I. T., Franseen, C. C., and Sweeney, A. R., Jr., *Proc Soc Exp Biol and Med.*, 1938, **39**, 365.

<sup>2</sup> Nathanson, I. T., and Rawson, R. W., *Proc Soc. Exp Biol and Med.*, 1939, **42**, 482.

<sup>†</sup> We are very grateful for the liberal quantities of testosterone propionate which were furnished us by Doctors Gregory Stragnell and Max Gilbert of the Schering Corporation, and by Dr. Ernst Oppenheimer of Ciba Co., under the trade names of Oreon and Perandren respectively.



The thyroid and parathyroid glands of each animal were fixed in Zenker's solution imbedded in paraffin, and representative sections were stained with phloxine methylene blue

Measurements of height of the acinar cells of the thyroid were made according to the method of Rawson and Starr,<sup>3</sup> in which 100 cells were measured in each gland. Mitoses were counted, using the technic of Brues and Marble.<sup>4</sup> At least 4000 cells were counted in each gland, and at least one entire section of each gland was examined

*Results* Table I presents the results of this experiment

It can be seen from the results presented in the table that testosterone propionate is capable of stimulating the thyroid and parathyroid glands of the intact immature female rat. The increase in mitotic activity especially in the first 96 hours seems particularly significant. This corresponds very closely with changes seen in the ovary during the same period of time. Increase in mitotic activity was noted even in the first 24 hours. After 96 hours the activity decreased, but in all instances the levels remained at the upper limits, or above those of the controls. In one animal (No. 109), which showed a high degree of activity, corpora lutea were noted in the ovaries. These were not seen in the ovaries of the other animals.

Increase in cell height could be demonstrated in the thyroid gland almost as soon as the increase in mitotic activity was noted. It can be seen that in the early stages of the experiment an increase in mitosis was associated with an increase in the size of the cell. However, there was no direct correlation between the number of mitoses and cell height.

In the control group no marked difference could be detected between the animals treated with colchicine and those that had none, although the mean cell height seemed to be slightly lower in the colchicine-treated group. One control animal (No. 110) deserves comment. In this rat there was an increase in mitosis and cell height in the thyroid, as well as stimulation of the follicles of the ovaries. Autopsy revealed a cyst of the pituitary gland.

It is noteworthy that in all groups mitotic activity in the thyroid and in the parathyroid gland of each animal was approximately equal.

The mechanism of this stimulation is not clear at present. Several possibilities seem tenable.

(a) Testosterone stimulates the thyroid and parathyroid glands directly

<sup>3</sup> Rawson, R. W., and Starr, P., *Arch. Int. Med.*, 1938, **61**, 726

<sup>4</sup> Brues, A. M., and Marble, B. B., *J. Exp. Med.*, 1937, **65**, 15

TABLE I.  
Effect of Testosterone on Mitosis and Cell Height of Thyroid Gland

No	Wt onset experiment	Wt when sacrificed	Age when sacrificed in days	Hr after testosterone	Mitoses thyroid per 10,000 cells	Mitoses para thyroid per 10,000 cells	Mean acinar cell height per 100 acini	Remarks
					Treated Group			
114	48	49	32	24	46	28	$4.24 \pm 0.004$	Colehucine
103	40.5	48.5	33	48	41	—	$3.82 \pm 0.004$	"
115	46	47	34	72	22	33	$4.22 \pm 0.004$	"
105	45	57	35	96	118	77	$4.19 \pm 0.005$	"
111	44	50.5	36	120	12	—	$4.27 \pm 0.004$	"
101	43.5	63.5	37	144	12	10	$4.15 \pm 0.004$	"
102	43.5	61	38	168	11	22	$3.06 \pm 0.004$	"
109	43.5	69	40	216	75	—	$4.29 \pm 0.004$	"
104	42	69	41	240	10	—	$4.20 \pm 0.004$	"
107	42	85	42	264	18	15	$4.05 \pm 0.004$	"
113	43	88	43	288	19	22	$4.34 \pm 0.004$	"
					Control Group			
116	40	52	35	—	8	11	$3.85 \pm 0.005$	No colehucine
117	45	53	35	—	2	—	$3.61 \pm 0.004$	Colehucine
110	46.5	54	34	—	36	—	$4.17 \pm 0.005$	" cyst pituitary
108	45.5	68	38	—	6	—	$3.58 \pm 0.004$	Colehucine
118	42	67	39	—	7	—	$3.64 \pm 0.004$	"
119	41	66	39	—	2	9	$3.74 \pm 0.004$	No colehucine
112	42	72	43	—	7	8	$3.66 \pm 0.004$	Colehucine
120	40	82	44	—	6	4	$4.03 \pm 0.005$	"
121	42	85	44	—	4	10	$3.74 \pm 0.004$	No colehucine

corpora lutea

(b) Testosterone stimulates the thyroid and parathyroid glands either by way of the anterior pituitary or through some other gland of internal secretion

The second thesis seems more plausible, especially since the changes noted so closely parallel those in the ovary which we have shown are stimulated by way of the hypophysis. Experiments are under way at present to clarify these points

*Conclusion* Testosterone propionate is capable of stimulating the thyroid and parathyroid glands of the intact immature female rat. This stimulation is manifested by increased mitotic activity and by histological evidence of functional activity

## 11320 P

### The Mechanism of Action of Heparin\*

MORRIS ZIFF AND ERWIN CHARGAFF

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It is known that heparin does not prevent the coagulation of a fibrinogen solution by purified thrombin<sup>1</sup>. It likewise is unable to prevent the conversion of isolated prothrombin to thrombin<sup>2, 3</sup>. To exert its powerful anticoagulant action, heparin requires an additional factor which is known to be present in blood, plasma, and serum<sup>1, 4, 5</sup>. This factor has been shown by Quick<sup>4</sup> to be contained in the serum albumin fraction.

We have investigated the activity of various components of the albumin fraction in producing the anticoagulant effect of heparin on the fibrinogen-thrombin system.

The *thrombin* was obtained by the method of Eagle,<sup>6</sup> and the fibrinogen was prepared in the usual manner by repeated precipita-

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\* This work has been supported by a grant from the John and Mary R. Markle Foundation

1 Howell, W. H., and Holt, E., *Am J Physiol*, 1918-1919, **47**, 328

2 Mellanby, J., *Proc Roy Soc (London)*, 1934, **B 116**, 1.

3 Quick, A. J., *Proc Soc Exp Biol and Med*, 1936, **35**, 391

4 Quick, A. J., *Am J Physiol*, 1938, **123**, 712

5 Brinkhous, K. M., Smith, H. P., Warner, E. D., and Seegers, W. H., *Am J Physiol*, 1939, **125**, 683

6 Eagle, H., *J Gen Physiol*, 1935, **18**, 531

tions with sodium chloride *Heparin* was used in the form of the pure sodium salt †

The activities of the albumin components studied are shown in Table I, which reproduces a typical experiment. The letters in the first column of this table stand for the following compounds: *A*, crystalline albumin from human serum, prepared by Dr. F. E. Kendall of the Research Division for Chronic Diseases, Welfare Island. We are greatly indebted to Dr. Kendall for different specimens of this substance. *B*, albumin from sheep plasma, prepared according to Howe.<sup>7</sup> *C*, fraction from human plasma insoluble at 55% saturation with ammonium sulfate, presumably containing the globoglycoid of Hewitt.<sup>8</sup> *D*, fraction from human plasma insoluble at 75% saturation with ammonium sulfate. *E*, fraction from human plasma insoluble at 100% saturation with ammonium sulfate. This fraction presumably contained the seroglycoid of Hewitt.<sup>9</sup> *F*, the supernatant solution after removal of *E*, dialyzed and concentrated by ultrafiltration.

All substances were dissolved in physiological saline and carefully adjusted to the neutral point. The pH measurements were performed by means of the glass electrode. The experiments were carried out at room temperature as follows: 0.1 cc of a thrombin solution (from human plasma), 0.06 cc of a 0.3% heparin solution, and 0.06 cc of the protein solution were mixed. Three minutes later

TABLE I  
Influence of Albumin Fractions on the Anticoagulant Effect of Heparin

	30"	3'	5'	10'	15'	20'	25'
A, (9 mg N/cc) without heparin	+						
„ with „	+						
A, (4.5 mg N/cc) without heparin	+						
„ with „	+						
B, (1.4 mg N/cc) without heparin	—	—	+				
„ with „	—	—	—	—	—	—	—
C, (1.2 mg N/cc) without heparin	+						
„ with „	+						
D, (2.7 mg N/cc) without heparin	—	—	—	+			
„ with „	—	—	—	—	—	—	—
E, (1.2 mg N/cc) without heparin	+						
„ with „	—	—	—	—	—	—	—
F, (2.2 mg N/cc) without heparin	—	+					
„ with „	—	—	—	—	—	—	—

+ = clot

† We wish to thank Hoffmann-La Roche, Inc., Nutley, N. J., for the heparin preparation used.

<sup>7</sup> Howe, P. E., *J. Biol. Chem.* 1921, **49**, 93.

<sup>8</sup> Hewitt, L. F., *Biochem. J.*, 1938, **32**, 26.

<sup>9</sup> Hewitt, L. F., *Biochem. J.*, 1936, **30**, 2229, 1937, **31**, 360.

0.2 cc of a fibrinogen solution was added to the mixture. Fibrinogen preparations from human and sheep plasma gave essentially the same results. In the control experiments 0.06 cc of saline were substituted for the heparin. The tubes were examined for clots at fixed intervals in order to avoid unnecessary agitation. The concentration of the protein solutions used in the experiments reproduced in Table I is expressed in mg N per cc of protein solution.

As shown in Table I, the crystalline albumin fraction even when tested in fresh solution is entirely inactive as a complement of heparin. The activity appears to reside in the most soluble fraction of the serum albumin. It might be mentioned that the albumin solutions prepared by Howe's method retained their activity for more than 50 days. It is not possible to state definitely whether the activity is due to a single component of the albumin fraction. Additional work will have to be carried out with respect to this question. A detailed report on this work and related aspects will be published at a later date.

It might be mentioned that occasionally individuals are encountered whose clotting time responds to heparin to a slight degree only. It will be of interest to see whether the heparin complement here discussed is lacking in the serum of these patients.

## 11321

## Chloride Excretion in Hypothyroidism

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Cutler, Power and Wilder,<sup>1</sup> using a standardized sodium chloride depletion test, have shown that the chloride concentration in the urine of patients with Addison's disease is significantly and consistently higher than in controls. These observations have been confirmed by others.<sup>2</sup> Six of 8 patients with clinical hypopituitarism, studied under similar conditions, have shown increased concentrations of chloride in the urine comparable to those found in patients with

<sup>1</sup> Cutler, H. H., Power, M. H., and Wilder, R. M., *J. Am. Med. Assn.*, 1938, 111, 117.

<sup>2</sup> Dryerre, H. W., *Edinburgh Med. J.*, 1939, 46, 267.

Addison's disease Symptoms suggesting those of the Addisonian crisis developed in 4 of the 6 patients with abnormal chloride excretion, and were promptly relieved by the administration of sodium chloride and adrenal cortex extract These observations were interpreted as evidence of the occurrence of chronic adrenocortical insufficiency in clinical hypopituitarism, presumably secondary to withdrawal of the adrenotropic anterior pituitary principle.<sup>3</sup>

Decourt<sup>4</sup> has recently reported lowered blood chloride levels in myxoedema and suggests that the thyroid gland may play a rôle in the regulation of chloride metabolism Lowering of the basal metabolic rate is a prominent characteristic of hypopituitarism, and is occasionally observed in patients with Addison's disease However, in such patients, the administration of thyroid substance for the purpose of raising the metabolic rate may precipitate the crisis of acute adrenal insufficiency<sup>5, 6</sup> Similar reactions have not been reported in myxoedema In the present study of a group of patients with primary hypothyroidism, normal values for serum chloride were found and a normal response to sodium chloride restriction was observed

Six patients with the clinical and laboratory characteristics of primary hypothyroidism were studied In 2 patients the hypothyroidism was post-operative In each instance there was a satisfac-

TABLE I  
Chloride Excretion in Hypothyroidism.

Patient	BMB	Urine chloride (as NaCl) 4-hr spec, 3d day Mg per 100 cc
E.E	—24	50
M.I.	—24	95
A.T.	—25	58
M.H.	—27	79
D.B.	—30	140
M.K.	—42	49
Controls, 17 cases		111
		35-244
Addison's disease, 8 cases*		487
		382-593
Hypopituitarism 6 cases		518
		364-690
2 cases		65, 240

\*Includes cases of Cutler, Power and Wilder<sup>1</sup>

<sup>3</sup> Stephens, D. J., *Am J Med Sci*, 1940, 100, 67

<sup>4</sup> Decourt, J., *Ann d med*, 1938, 44, 133

<sup>5</sup> Means, J. H., *The Thyroid and Its Disorders*, J. B. Lippincott Co., Philadelphia, 1937, p. 530

<sup>6</sup> Lerman, J., and Salter, W. T., *Endocrinology*, 1939, 25, 712

tory response to the subsequent administration of thyroid substance. The standard procedure described by Cutler, Power and Wilder<sup>1</sup> was used. The chloride concentration of a 4-hour urine specimen collected on the third day of chloride restriction in each of the 6 patients is shown in Table I. In each case, this value falls well within the range which has been found in control subjects. No significant change was observed in the serum concentration of chloride, the carbon dioxide combining power or the blood non-protein nitrogen. None of the patients experienced any untoward symptoms during the period of sodium chloride restriction.

*Conclusions* Six patients with primary hypothyroidism, in whom chloride excretion was studied during periods of sodium chloride restriction, showed no evidences of limitation of adrenocortical function.

## 11322

## Dissociation of Pneumococcus by Radon Irradiation

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Although extensive studies have been made on the bactericidal action of various rays emanated from radio-active substances<sup>1</sup> very little attention has been paid to the cultural and morphological changes that may follow such irradiation. Spencer<sup>2</sup> first called attention to the possibility of producing in a certain number of instances, distinct and stable morphological variants by exposing *Streptococcus hemolyticus* and *E. typhi* to the gamma rays of radium. While some changes were noted in the cultural characteristics of these variants, few details were given. It has occurred to us that in addition to the morphological changes observed by Spencer, variations in cultural characteristics, serological specificity, and virulence might also result from irradiation. A comparison with another dissociant obtainable by the conventional method seems desirable. For these purposes, we have chosen the pneumococcus in our studies as the dissociants of pneumococcus are well known and readily ob-

<sup>1</sup> Spencer, R. R., *Public Health Report* (Wash., D. C.), 1934, **49**, 183

<sup>2</sup> Spencer, R. R., *Ibid.*, 1935, **50**, 1642

tainable In the present communication, the effect of non-lethal *gamma* rays from radon tubes on Type I pneumococcus is described, and the dissociants thus obtained are compared with those produced by immune serum alone

A virulent culture was plated and 10 colonies were picked in order to allow for the possible presence of some "radon-resistant" forms in the original culture The organisms were grown in test tube 5.5 x 1.5 cm containing 2 cc liver-digest broth containing 1% glucose After inoculation, radon tubes or bulbs of about 150 millicurie strength, encased in a brass capsule one mm in thickness, 6 mm in diameter and 30 mm in length, was placed in the same tube In this way, *gamma* rays only were obtained The tube was then incubated at 37°C Every 48 hours the culture was transferred into a new medium with the brass capsule containing radon tubes or bulbs Every 24 hours the radon in tubes or bulbs was replenished to the initial strength of about 150 millicurie Plates were made every day and colonies carefully studied Occasionally, the culture was found to be contaminated, then the transfer was continued by picking some colonies from the plate made from the last irradiated culture At the same time the following parallel experiments were run (1) Culture with brass capsule without radon (control) (2) Culture grown in broth containing 5% homologous immune serum, and (3) Culture grown in broth containing 5% homologous serum and irradiated with radon at the same time

*Results* As the results with 2 series of experiments were essentially identical, only one of them is described in detail After the 2nd transfer, colonies of the irradiated culture were found to be larger and more convex and mucoid than the controls, but returned to normal after another 3 transfers From then until the 10th transfer, only smooth colonies were obtained Five of such from the 10th transfer were inoculated into broth and the organisms were found to be fully virulent Suddenly after one more transfer, striking changes were brought about the colonies were all small, dry and extremely rough, but were completely soluble in bile and agglutinable by Types 2 and 3 sera Twenty out of about 200 colonies obtained from the 11th irradiated culture were transferred to broth and tested for virulence It was found that the sedimented organisms derived from as much as 20 cc of broth culture failed to kill white mice The surviving animals were susceptible to the virulent organisms These dissociants remained stable after weekly transfers for at least 6 months At no time was a smooth colony observed in any of the subcultures Attempts to revert 10 such colonies by the



mouse-method<sup>3</sup> were negative. It thus appeared that the dissociation was complete. Similar results were obtained with the second series, the only difference being that this sudden change in cultural characteristics and virulence took place after the 13th transfer.

The result from the control experiment was as follows. No change was observed with cultures containing brass capsule only, thus ruling out the possible dissociative effect of brass. The cultures grown in 5% immune serum broth also dissociated into rough forms. The following differences, however, were noted: (1) The pneumococcus grown in immune serum showed gradual changes from S to R while that seeded in the presence of radon irradiation exhibited sudden changes. (2) The loss of virulence of the serum-treated organisms appeared less complete. Even after the 20th transfer, 2-3 cc of broth culture could kill mice from which fully virulent pneumococcus could be recovered. In the presence of immune serum, a fewer number of transfers and a shorter period of irradiation were required to bring about dissociation by radiation. Thus in one experiment, only 5 daily transfers were necessary to produce 95% rough colonies from the radiated cultures. They possessed all the characteristics of those produced by radon alone.

From the above observations, it seems that irradiation with *gamma* rays obtained from radon can regularly induce a change of smooth to rough form of pneumococcus so complete that it was impossible to revert the dissociants to the primary state. Furthermore by a combined action of immune serum and irradiation, it has been possible to achieve the same results but in a much shorter period of irradiation. This latter observation may offer a plausible explanation of the mode of therapeutic action of X-rays in cases of pneumococcal lobar pneumonia.<sup>4</sup>

*Summary* Type I pneumococcus could be dissociated by *gamma* ray radiation. The rough forms appeared suddenly, becoming totally avirulent and irreversible. This dissociative change could be achieved in a shorter period of irradiation by the addition of immune serum.

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<sup>3</sup> Griffith, F., *J. Hyg.*, 1928, **27**, 113.

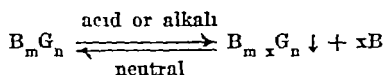
<sup>4</sup> Scott, W. R., *Radiology*, 1939, **33**, 331.

# Recovery of Antigen from Type I Pneumococcus Immune Precipitate

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In previous reports<sup>1 2</sup> from this laboratory, it has been shown that the liberation of antibody from immune precipitate by the action of acid or alkali is due to a shift of equilibrium as follows



Where G = antigen, B = antibody and  $B_m G_n$  = immune precipitate formed in neutral solution. Upon treatment with acid or alkali, a part of the antibody  $x B$ , is set free from the precipitate  $B_m G_n$ , leaving behind an acid or alkaline precipitate,  $B_m x G_n$ . Since the acid or alkaline precipitate,  $B_m x G_n$  has a higher antigen content than the neutral precipitate,  $B_m G_n$ , it should, when separated from the free antibody,  $x B$ , liberate some antigen upon neutralization. The purpose of the present study is to test this point.

Portions of Type I Pneumococcus horse immune precipitate (4 mg N each) were treated with dilute HCl or NaOH of different concentrations in the presence of 1% NaCl according to the technic reported in a previous communication<sup>2</sup>. After centrifuging the supernatants were used for pH determinations. The acid or alkaline precipitates were evenly suspended in 2 cc of normal saline and neutralized. The neutralized suspensions were centrifuged. One cc portions of the neutralized supernatants were mixed with 1 cc of the same homologous antiserum, which was used in preparing the original immune precipitate, while the remaining portions were mixed with normal serum as control. The mixtures were incubated at 37°C for 2 hours and then chilled at 0°C overnight. Precipitation occurred in some of those tubes containing antiserum, but not in the tubes with normal serum. That the precipitation was caused by a combination of antigen and antibody and was not a reprecipitation of dissolved immune precipitate by mass action effect was shown by the fact that saline washings of Type I Pneumococcus

<sup>1</sup> Liu, S C, and Wu, H, *Chinese J Physiol*, 1938, **13**, 449

<sup>2</sup> Liu, S C, and Wu, H, *Proc Soc Exp Biol and Med*, 1939, **41**, 144

TABLE I  
Recovery of Antigen from Type I Pneumococcus Immune Precipitate at Different pH's in the Presence of 1% NaCl  
Temperature = 23°C

pH	2.38	2.68	3.02	3.38	3.88	—	9.72	9.86	10.22	10.65	10.92
Precipitate,* mg N	0.154	0.084	0.042	0	0		0	0	+	+	+
Polysaccharide recovered,† mg	0.20	0.10	0.05	0	0		0	0	+	+	+

\*Precipitate formed by the recovered antigen with 1 cc antiserum

† Read from a control precipitation curve

0 = No precipitation.

+ = Definite precipitation but the amount was too small to be estimated

immune precipitate, when mixed with the homologous antiserum, gave no precipitation under the same experimental conditions

The precipitates were centrifuged at 0°C and washed twice with 1 cc saline also at 0°C. The N contents of the precipitates were determined by micro-Kjeldahl method. A control precipitin experiment was done by adding known amounts of the same lot of Type I Pneumococcus polysaccharide to the same lot of antiserum as that used in preparing the original immune precipitate under the same experimental conditions. The amount of precipitate N was plotted against the amount of polysaccharide. The amount of polysaccharide recovered at different pH's in the recovery experiment was then read from the control precipitin curve. The results are shown in Table I. It will be noted that antigen was recovered only in comparatively more acid solutions, where the recovery of antibody was previously shown<sup>2</sup> to be over 50%. When the pH was higher than 3.38, the amount of antigen recovered was too small to be estimated accurately.

**Summary** Antigen was recovered from Type I Pneumococcus immune precipitate from which some antibody had been removed by treatment with acid. This experiment substantiates our previous finding that the recovery of antibody from immune precipitate by the action of acid or alkali is due to a shift in the antigen-antibody equilibrium. The present finding also suggests a possible method for the isolation of pure antigen which may be useful when it cannot be obtained otherwise.

## 11324

### Further Studies on Type-Specific Protein of *Corynebacterium diphtheriae*

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In a previous study<sup>1</sup> it was found that a serologically active, type-specific protein could be prepared by mild alkaline extraction at low temperature from the well-known Park 8 strain. In view of this finding it seems of interest to extend this observation to other types of *C diphtheriae* in order to determine (1) whether the method of

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<sup>1</sup> Wong, Sam C, and T'ung, T, *Proc. Soc. Exp. Biol. and Med.*, 1939, **42**, 824

extraction has a general application and (2) whether the protein is responsible for type-specificity of all types

**Method** The organisms employed for the present investigation were selected at random from the representative types of Sia and Huang's scheme of classification of *C diphtheriae*<sup>2</sup> Seven strains belonging to 5 serological types and including the cultural types of gravis, intermediate and mitis were used. All but one (strain No 7) were virulent. The growing of the organisms, the method of preparing the protein antigens and the production of immune sera with the 7 strains were the same as those previously outlined<sup>1</sup>. In addition, immune sera of the following strains were also prepared: 8, 9, 10, 11. In all instances 2 rabbits for each strain, making a total of 22, were used. The precipitin ring test was performed at room temperature by carefully laying various dilutions of the antigen over a constant amount of undiluted immune serum and the results, read 2 hours later. The protein content of each preparation was estimated by determining the total nitrogen with the Micro-Kjeldahl method. The nitrogen value was multiplied by the protein factor 6.25 to give the concentration in milligrams. In the table one mg of protein is expressed as 1/1000 dilution.

In the course of this investigation several difficulties arose. In the first place the diphtheria bacilli are essentially poor antigens. While a type-specific agglutinating serum could be obtained generally in about 3-4 weeks of immunization in rabbits, a precipitin serum either for the proteins or for the polysaccharides may require several months. In 2 instances (strains 2 and 4) antibodies could not be detected by various dilutions of the polysaccharide although precipitins were present when tested with the protein antigens at high concentrations. Satisfactory sera for strains 2, 4 and 5 were obtained only after 6 months of immunization. New animals were used for a few of the strains without any apparent success. In the second place, sera of rabbits immunized with *C diphtheriae* invariably acquired anticomplementary properties. This fact renders the complement-fixation test inapplicable for the detection of type-specific antibodies. In the third place, serological activities of the proteins varied with different preparations. At times it was almost impossible to decide whether the protein was inactive or whether the immune serum was unsuitable. In general, however, the potency of an immune sera for the specific protein antigen may be estimated by its precipitin titer for the polysaccharide. The higher the titer for the

<sup>2</sup> See R. H. P. and Huang, C. H., *Ibid.*, 1939, **41**, 348

latter the more suitable is the serum for the demonstration of the type-specific antigen.

**Chemical Studies** All the protein preparations obtained gave the usual color reactions for proteins (biuret, xanthoproteic Hopkins-Cole and Millon). They could be precipitated by neutral salts such as ammonium sulfate and sodium sulfate, by heavy metals such as copper sulfate or lead acetate, and by trichloroacetic and tannic acids. The Molisch test for polysaccharide in 1/500 dilution of the protein was negative. Extraction of proteins with ether did not destroy type-specificity but their serological activities were reduced. This indicates that the specific component is not a lipid-complex.

In our previous report<sup>1</sup> we found that the type-specific protein prepared from Park 8 was heat-labile, being converted into a group specific protein upon heating at 56°C for 30 minutes. Subsequent study on this and other type-specific proteins, however, showed that this observation has no general application and appears to vary with different preparations which in turn depend upon a number of undetermined factors. It seems that the more active the preparation the conversion is more likely to occur although complete destruction of serological activity by heat has yet to be observed. Indeed, all the protein preparations employed in the present study in concentrations of 5-10 mg could withstand heating at 122°C for 20 minutes without alteration of type-specificity nor diminution of serological activity. However, the phenomenon of type to group-specific conversion could be demonstrated with certain preparations particularly from some strains (Park 8 and No. 3). Furthermore all proteins prepared by 1% potassium hydroxide extraction of organisms in boiling water bath are group-specific although the serological activity in general

TABLE I  
Type-specificity of Proteins as Shown by Precipitin Reactions.

Serum Type		Protein						
		1	2	3	4	5	6	7
1	D41	1 100,000	1 5,000	—	—	—	—	—
2*	D41	1 5,000	1 1,000	—	—	—	—	—
3	D43	—†	—	1 100,000	1 5,000	—	—	—
4	D43	—	—	1 10,000	1 1,000	—	—	—
5	D14	—	—	—	—	1 5,000	—	—
6*	6287	—	—	—	—	—	1 10,000	—
7*	X	—	—	—	—	—	—	1 5,000
8	D25	}	—	—	—	—	—	—
9	D30							
10	D40							
11	1219							

\*Sera which have been previously absorbed

†Indicates negative to 1/500 dilution of the protein.

extraction has a general application and (2) whether the protein is responsible for type-specificity of all types

*Method* The organisms employed for the present investigation were selected at random from the representative types of Sia and Huang's scheme of classification of *C diphtheriae*<sup>2</sup> Seven strains belonging to 5 serological types and including the cultural types of gravis, intermediate and mitis were used All but one (strain No 7) were virulent The growing of the organisms, the method of preparing the protein antigens and the production of immune sera with the 7 strains were the same as those previously outlined<sup>1</sup> In addition, immune sera of the following strains were also prepared 8, 9, 10, 11 In all instances 2 rabbits for each strain, making a total of 22, were used The precipitin ring test was performed at room temperature by carefully laying various dilutions of the antigen over a constant amount of undiluted immune serum and the results, read 2 hours later The protein content of each preparation was estimated by determining the total nitrogen with the Micro-Kjeldahl method The nitrogen value was multiplied by the protein factor 6.25 to give the concentration in milligrams In the table one mg of protein is expressed as 1:1000 dilution

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<sup>2</sup> Sia, R. H. P., and Huang, C. H., *Ibid.*, 1939, 41, 348

*Conclusion* From the observations made on the chemical and immunological studies of the polysaccharide, lipoid, and protein fractions obtained from representative serological and cultural types it is justifiable to conclude that the alkali-soluble protein is the cellular constituent responsible for type-specificity in *C diphtheriae*

11325

## Thyroid Enlargement Following Liver Feeding in Rats

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That the feeding of liver to animals produces an enlargement of the thyroid gland was reported by Hunt,<sup>1</sup> who found that the thyroid of mice fed on a diet of oatmeal and liver was larger and more vascular than of those on a diet of eggs, crackers and milk. Marine<sup>2</sup> produced a hyperplasia of the thyroid in brook trout by feeding them with liver and heart. Burget<sup>3</sup> found that rats kept under hygienic conditions and fed a high protein diet, consisting of fresh liver and lean beef mixed with a little oatmeal or bread crumbs, developed a hyperplasia of the thyroid. All the above reports of enlargement of the thyroid were interpreted as due to the high protein diet.

Recently, Remington<sup>4</sup> reported that in a low iodine goiter-producing diet, the replacement of a part of the wheat gluten by dried pig liver aggravated the degree of goiter, while purified casein prevented goiter formation, and yeast had no effect. This led Remington to believe that the liver in his goiter-producing diet was responsible for the enlargement of the thyroid. The writer (Hou<sup>5</sup>) in the same year reported that rats fed on a soybean-millet diet with dried liver powder as the only source of animal protein developed a marked hypertrophy of the thyroid gland. The enlargement was over 10 times when the animal was fed on the millet and liver diet for over a year. It becomes of great interest to know which constituent or constituents

<sup>1</sup> Hunt, Reid, *J Am Med Assn*, 1911, 57, 1032

<sup>2</sup> Marine, D, *J Exp Med*, 1914, 10, 70

<sup>3</sup> Burget, G E, *Am J Physiol*, 1917, 44, 492

<sup>4</sup> Remington, R. E., *J Nutrition*, 1937, 13, 223

<sup>5</sup> Hou, H C, *Chinese J Physiol*, 1936, 10, 659, 1937, 12, 488



was low. The mechanism underlying these reactions is being studied.

**Serological Studies** The results are presented in Table I. It is to be noted that all sera except strains 2 and 4 used in these studies were very potent when tested with the polysaccharide, precipitin titers ranging from 1:200,000 to 1:500,000 being observed. An analysis of the data reveals several points of interest. In the first place proteins from certain strains appear to be more active in serological test than others. For example antigens prepared from strains 1, 3, 6, 7 gave a specific reaction with the homologous type sera in dilutions of 1:50,000 to 1:100,000 while those from strains 2, 4 and 5 reacted in only 1:5000. It is conceivable that the marked difference in serological activities of the various antigens may be due either to denaturation of the antigen during preparation or to unsuitable serum. In the second place none of the proteins reacted with heterologous type-sera produced with strains 8, 9, 10 and 11. In the third place all the protein antigens are strictly type-specific with the exception of those from strains 6 and 7. Even here there was a quantitative difference, the protein reacting with the homologous serum to a much higher titer. In order to show the type-specificity, reciprocal absorption tests were carried out between the undiluted immune sera of strains 2, 6 and 7 and the organisms. The results showed that the common precipitins were promptly removed. The titers of the resulting type-specific sera, however, were significantly lowered when retested with the homologous protein. In case of sera of strains 6 and 7 there was a ten-fold drop in the precipitin titer. This may explain the marked cross reactions observed in the slide agglutination test with these strains.

**Comment** The result of the present study shows clearly (1) that the method, consisting of alkaline extraction of bacilli previously defatted, is applicable to all the types studied and (2) that the protein is the cellular component responsible for type-specificity. For the demonstration of the latter point, serum from superimmunized animals in general is necessary. Furthermore, if marked cross reaction occurs in the agglutination test it is also likely to occur with the protein antigens. The difference, however, is quantitative and the common antigenic factor could be easily removed by reciprocal absorptions between immune serum and the heterologous organism. It may be mentioned that the third cellular component of *C. diphtheriae*, the lipoids, obtained from all the 7 strains are, like the polysaccharides,<sup>3</sup> group-specific. This is in agreement with the observation previously reported on Park 8.<sup>1</sup>

enlargement of the thyroid. Furthermore, when the liver had been extracted with alcohol (diet A7b) its feeding did not induce any enlargement of the thyroid. On the other hand, the inclusion of the residue from the alcoholic extract of liver in the diet containing dried beef (A7c) caused the thyroid to increase in size to a degree approximating the enlargement due to the dried liver diet (A7).

It appears, therefore, that the factor which causes an enlargement of the thyroid in rats lies in the alcohol-soluble portion of the dried liver. The enlargement as shown by histological examination was general in character with the organ a little more vascular and cellular than normal. Further experiments are being carried out to determine the actual substance present in the alcohol-soluble portion of liver which causes the enlargement of the thyroid.

### 11326 P

#### Cultivation of Virus of Encephalitis (St. Louis Type) on Agar Tissue Medium

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Although the virus of encephalitis of St. Louis type has been successfully cultivated by Maitland technic and on the chorio-allantoic membrane of developing chick<sup>1-4</sup> the titer obtained in these cultures has been invariably low. None of the cultures showed a titer as high as  $10^6$  even after repeated passages. Recently Kawakita<sup>5</sup> has reported successful cultivation of Japanese encephalitis virus in a special medium containing chick allantoic fluid. It has occurred to us to attempt to cultivate the virus of St. Louis type on the agar-tissue medium which has been found to be satisfactory for the growth of various types of rickettsiae.<sup>6-7</sup> The results of such a study together

<sup>1</sup> Sterverson, J. T., and Berry, G. P. *Science* 1935, **82**, 596.

<sup>2</sup> Harrison, R. W., and Moore, E., *Am. J. Path.* 1937, **13**, 361.

<sup>3</sup> Schultz, E. W., Williams, G. F., and Hetherington, A., *Proc. Soc. Exp. Biol. and Med.* 1938, **38**, 799.

<sup>4</sup> Smith, M. G. *Proc. Soc. Exp. Biol. and Med.* 1939, **40**, 191.

<sup>5</sup> Kawakita, Y., *Jap. J. Exp. Med.* 1939, **17**, 211.

<sup>6</sup> Zinsser, H., FitzPatrick, F. K., and Wei, H., *J. Exp. Med.* 1939, **69**, 179.

<sup>7</sup> Zia, S. H., Liu, P. Y., and Pang, K. H. *Chinese Med. J.*, 1938, **54**, 547.

of the diet is or are responsible for the enlargement of the thyroid. The following series of experiments were accordingly carried out.

Albino rats of the Wistar strain were selected with reference to litter mates and sex when about one month old. Five rats, each from a different litter, were put into one group. The litter mates were put into 3 other groups. The 4 groups were given 4 different diets as follows:

A7	Soybean Flour	450	A7a	Soybean Flour	450
	Millet	500		Millet	500
	Sodium Chloride	20		Sodium Chloride	20
	Dried Ox Liver Powder	30		Dried Beef	30
				Carotene (0.008 mg per rat per day)	
				Ostelin	2 cc
A7b	Soybean Flour	450	A7c	Soybean Flour	450
	Millet	500		Millet	500
	Sodium Chloride	20		Dried Beef	30
	Dried Ox Liver (extracted with Alcohol)	30		Sodium Chloride	20
	Carotene (0.008 mg per rat per day)			Residue of Alcohol Extract of Ox Liver (from 30 g of dried liver)	
	Ostelin	2 cc		Ostelin	2 cc

The rats were kept on these diets for 3 months and then sacrificed. The thyroid glands were weighed with a micro-analytical balance immediately upon their removal from the body. The weights of thyroid glands of the various groups are shown in Table I.

It will be noted that rats fed on diet A7 had the largest thyroid, next rats on A7c, then those on A7a. Rats fed on A7b had the smallest thyroid which is within the normal range of thyroid weight. From these results it appears that the dried ox liver was the constituent in the diet which caused the marked enlargement of the thyroid since the substitution of liver by dried beef resulted in only a slight

TABLE I  
Influence of Liver and Liver Extract on Thyroid Gland.

	A7		A7a		A7b		A7c (Alcoholic extract of liver)	
	(Dried ox liver)		(Dried beef)		(Extracted liver)			
	Body wt g	Thyroid mg	Body wt g	Thyroid mg	Body wt g	Thyroid mg	Body wt g	Thyroid mg
	127	58.7	96	14.8	57	9.2	110	31.8
	98	53.8	88	19.0	150	16.0	120	38.3
	138	55.5	93	24.3	164	17.0	130	35.4
	108	33.8	156	42.9	158	13.5	140	50.0
	90	29.7	113	23.5	147	18.1	111	28.3
Avg	112	46.3	109	24.9	135	14.8	122	36.7
Thyroid mg/100 g Body wt.	41.3		22.8		10.9		30.1	

methods Attempts have been made to utilize the cultured virus for the preparation of immunizing vaccine but so far only equivocal results have been obtained With the recent report of Kurotchkin<sup>8</sup> on the successful cultivation of vaccinia virus on the same medium, further trials with some other viruses seem to be indicated

## 11327 P

## Ultraviolet Absorption Spectrum of Cytochrome C

GEORGE I LAVIN, CHARLES L HOAGLAND AND S M WARD  
(Introduced by O T Avery )

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Dixon Hill and Keilin<sup>1</sup> published the ultraviolet absorption curve of cytochrome c A sharp maximum was found at about 4150 Å Other than a plateau in the region 2600-2900 Å no other structure was detected Roche and Benevent<sup>2</sup> also found a similar curve

The cytochrome c used was prepared from fresh beef heart by the method of Keilin and Hartree<sup>3</sup> Tetrapyrrolic iron was determined by the dipyrindyl technic of Hill,<sup>4</sup> following the treatment of cytochrome c with alkali and hydrogen peroxide Several lots were assayed for the hemin iron content with an average value of 0.39% The theoretical value is around 0.4%<sup>5</sup>

Since it is known that the absorption curves obtained with the line source and photometer may fail to show all the details of the bands<sup>6</sup> photographs were taken with the continuous light of the hydrogen discharge tube and a small Hilger quartz spectrograph The spectrum obtained is drawn in Fig 1a The band at 4150 Å is seen, also other bands not clearly indicated in the curve The band in the region of 3500 Å is rather diffuse as are the other bands in the protein region Not shown in the illustration are bands in the region 2550-2600 Å These are of such intensity that it is difficult to obtain their exact wave length<sup>6</sup>

<sup>8</sup> Kurotchkin, T J, *Proc Soc Exp Biol and Med*, 1939, **41**, 407

<sup>1</sup> Dixon, M, Hill, R, and Keilin, D, *Proc Roy Soc B*, 1931, **100**, 29

<sup>2</sup> Roche, J, and Benevent M T, *Bull Soc Chim Biol* 1935 **17**, 1473

<sup>3</sup> Keilin, D, and Hartree, E F, *Proc Roy Soc B* 1937 **122**, 298

<sup>4</sup> Hill, R, *Proc Roy Soc B*, 1930 **107** 205

<sup>5</sup> Theorell, H, *Science* 1930, **60**, 67

<sup>6</sup> Lavin, G I, and Northrop J H *J Im Chem Soc* 1935 **57** 874

with a preliminary study on the immunizing value of vaccine prepared from these cultures are hereby communicated

The virus, Webster No. 3, which has full virulence for mice, was propagated in the following fashion. Infected mouse brain, diluted with serum-Tyrodé solution mixture (1:5) to 10% by weight was filtered through Seitz E-K pad after brief centrifugalization. A small amount of the filtrate was inoculated on to a small piece of young mouse embryonic tissue in a sterile Petri dish. These were minced together and after being allowed to stand for 15 to 20 minutes at room temperature, the finely cut tissue bits were carefully laid on the surface of agar slants employed for the cultivation of rickettsiae. These cultures were incubated at 37°C, and transferred every 5-8 days. By this method, the virus has been carried for over 40 generations in a period for more than 10 months. The activity of the virus for Swiss mice by intracerebral injections of different generations was found to be as follows:  $10^{-6}$  for the sixth,  $10^{-6}$  for the sixteenth,  $10^{-7}$  for the twenty-first and  $10^{-6}$  for the thirty-fourth generations of cultures. It was also found that as little as  $10^{-6}$  or  $10^{-7}$  dilution of the filtrate of infected mouse brain or only a few MLD for mice could successfully initiate cultures on agar tissue medium. These observations showed conclusively that there has been satisfactory multiplication of the virus on embryonic tissue lying on the surface of agar containing serum and Tyrodé solution, and that the titer in each generation might reach that of infected mouse brain. Furthermore, relatively small amount of virus may initiate these cultures which suggests that the cultural method might be applicable in the isolation of virus.

Vaccine was prepared by grinding the tissue bits collected from several tubes and preserved with 0.1% formalin. Each cc of the vaccine contained materials which had 10,000 MLD for mice before inactivation. Immunization experiments have been attempted with 10 mice inoculated intraperitoneally at 3 days' interval with 2.5 cc and another 10 mice with 1.5 cc of the vaccine. One week after the last injection, the mice received intracerebral injections of 1,000 MLD for mice. So far none of the animals that received culture vaccine survived the infecting dose. However, the possibility of employing this cultural method for the preparation of successful vaccine remains to be further exploited.

*Comment.* In contrast to the results of previous workers, it has been possible, by means of the agar tissue medium, to cultivate the virus of encephalitis of St. Louis type, for over 40 generations with no decrease in its virulence for mice. It has also been possible to obtain higher titer of multiplication than was possible by previous

stitched to the two nearest corners of the platform with silk ligatures. An incision about 2 cm long was then made through a part of the uterine wall which was free of placental attachments, the membranes ruptured and the hind-quarters of the foetus delivered until the umbilical cord came into view. If the foetus was lying in a favorable position, it was possible to expose the umbilicus with the umbilical vessels without delivering any part of the foetus. The umbilical cord was then ligatured in 2 places as close to the umbilicus as possible, divided, and the placental end delivered through the wound in the uterine wall. Thereafter the foetus was replaced within the uterus and the uterine wall stitched up again, leaving only the free end of the umbilical cord protruding. With practice it was possible to perform this operation without any appreciable loss of amniotic fluid, so that the intrauterine pressure was not materially altered.

The vessels of the cord were cleaned, a cannula was inserted into the umbilical artery and perfusion of the placental circulation immediately started by means of a small Dixon pump. The perfusion apparatus was the same as was used by Robson and Schild.<sup>1</sup> Another cannula was rapidly inserted into the umbilical vein to collect the perfusion fluid and thus prevent distention or clotting within the placental circulation.

The inflow perfusion pressure was recorded on the kymograph by a mercury manometer. The blood passing from the pump to the umbilical artery was kept at body temperature by passing the rubber tubing, which connected the two, through the heating apparatus of the operating table. The venous return from the umbilical artery was allowed to flow by gravity through the cannula and rubber tubing to the reservoir of the pump. The dead space of the perfusion apparatus (about 20 cc) was filled either with saline or with blood taken from the maternal circulation. Coagulation was prevented by means of repeated additions of heparin to the contents of the reservoir. Approximately 4 mg of heparin dissolved in 1 cc Ringer-Locke solution were required every half to one hour. The cannulae were maintained in position by skewering them to the cork platform and this insured that there was no kinking of or traction upon the umbilical vessels.

The contractions of the part of the uterus containing the foetus were simultaneously recorded by means of a Cushny myograph. By the use of a small celluloid window and pads of cotton wool the abdominal contents were maintained at a normal body temperature.

The minute volume of the circulation in the full-term foetus has been estimated to be of the order of 0.1 cc/g weight of the foetus in

<sup>1</sup> Robson, T. M., and Schild, H. O., *J. Physiol.* 1938 92 9.

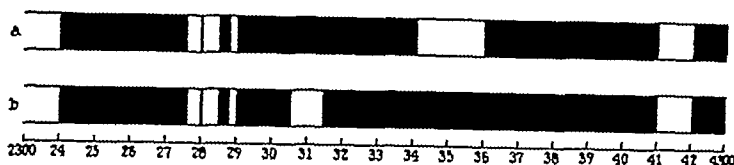


Fig. 1

When cytochrome *c* is treated with sodium formaldehyde sulfoxylate ( $\text{CH}_2\text{OHOSONa}$ ) the band at 3500 Å disappears and a new band appears at about 3100 Å, as depicted in Fig 1b. At the same time the well characterized band of reduced cytochrome *c* becomes apparent in the visible at 5500 Å. Experiments are now in progress to determine the possible reversibility of this process and the complete significance of the above data is being further investigated.

11328

### A Technique for the Perfusion of the Foetal Placental Circulation \*

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*From the Department of Pharmacology, University of Edinburgh*

Investigation of the various factors which control the placental circulation has necessarily been limited owing to the experimental difficulties involved. It is suggested that the following technic may offer a line of approach to some of the problems involved.

**Methods** The experimental animals used were cats or bitches which were in an advanced stage of pregnancy. Rabbits were found to be unsuitable owing to the friability of the placental vessels. Anesthesia was induced with open ether, and maintained with chloralose given intravenously in a dose of 100 mg/kilo body weight. Both vagi were cut, and the carotid blood pressure recorded by means of a mercury manometer. The abdomen was opened with as short an incision as was compatible with adequate access, and a part of the uterus containing one foetus brought up into the wound. A small cork platform approximately 5 cm square was approximated to the section of uterus exposed and the uterine peritoneum loosely

\* The expenses of this investigation have been defrayed by a grant from the Medical Research Council.

stitched to the two nearest corners of the platform with silk ligatures. An incision about 2 cm long was then made through a part of the uterine wall which was free of placental attachments, the membranes ruptured and the hind-quarters of the foetus delivered until the umbilical cord came into view. If the foetus was lying in a favorable position it was possible to expose the umbilicus with the umbilical vessels without delivering any part of the foetus. The umbilical cord was then ligatured in 2 places as close to the umbilicus as possible, divided, and the placental end delivered through the wound in the uterine wall. Thereafter the foetus was replaced within the uterus and the uterine wall stitched up again leaving only the free end of the umbilical cord protruding. With practice it was possible to perform this operation without any appreciable loss of amniotic fluid, so that the intrauterine pressure was not materially altered. The vessels of the cord were cleaned, a cannula was inserted into the umbilical artery and perfusion of the placental circulation immediately started by means of a small Dixon pump. The perfusion apparatus was the same as was used by Robson and Schild.<sup>1</sup> Another cannula was rapidly inserted into the umbilical vein to collect the perfusion fluid and thus prevent distention or clotting within the placental circulation.

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The contractions of the part of the uterus containing the foetus were simultaneously recorded by means of a Cushny myograph. By the use of a small celluloid window and pads of cotton wool the abdominal contents were maintained at a normal body temperature. The minute volume of the circulation in the full-term foetus has been estimated to be of the order of 0.1 cc/g weight of the foetus in

<sup>1</sup> Robson, J. M. and Schild H. O., *J. Physiol.* 1938 92, 9



goats (Barcroft, *et al*<sup>2</sup>) The weight of the foetus of the cat in the later stages of pregnancy is about 50 g In order to insure an adequate blood flow through the placenta the Dixon pump was adjusted to deliver about 8 cc/min, and at this rate the perfusion pressure was usually approximately 80 mm Hg

At the end of each experiment the adequacy of the perfusion was checked by injection of methylene blue into the venous cannula, and subsequent examination of the placenta In all cases it was found that the perfusion had been satisfactory

*Results* When 1  $\mu$ g of acetyl choline was injected intravenously into the maternal circulation it caused a small and transitory fall in the maternal blood pressure, but had no effect upon either the uterine contractions or the perfusion pressure Ten  $\mu$ g caused a greater fall in the maternal blood pressure, but again did not affect either uteri contractions or perfusion pressure When 1  $\mu$ g of acetyl choline was injected into the arterial cannula of the perfusion system, a slight and transitory rise occurred in the perfusion pressure, and this effect was greater and more prolonged when the amount of acetyl choline injected was increased to 100  $\mu$ g In neither case was there any demonstrable effect upon either the uterine contractions or the maternal blood pressure

Adrenalin was injected into the perfusion system in doses of 1 to 100  $\mu$ g In one experiment a sustained rise in perfusion pressure followed the injection of 10  $\mu$ g of adrenalin, but in none of the other experiments did the administration of the drug produce any effect on the perfusion pressure the maternal blood pressure, or the uterine contractions

0.1-1.0 unit pituitary (posterior lobe) extract injected into the maternal circulation caused a prolonged rise in the maternal blood pressure, and an increase in both the rate and the amplitude of the uterine contractions with a consequent increase in the rate and amplitude of the variations in the perfusion pressure This is illustrated in Fig 1 When 1 unit was injected into the perfusion system it caused a sustained rise in perfusion pressure, with, in one case an increased rate of uterine contractions In no case was any effect on the maternal blood pressure noted

A constant observation in these experiments was that cyclical variations in perfusion pressure occurred synchronously with the uterine contractions This is in contradistinction to the findings during artificial perfusion of the maternal-uterine circulation, where uterine tone and motility have no appreciable effect upon the perfusion pressure (Robson and Schild<sup>1</sup>)

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<sup>2</sup> Barcroft, J, Kennedy, J A, and Mason, M F, *J Physiol*, 1939, **95**, 269

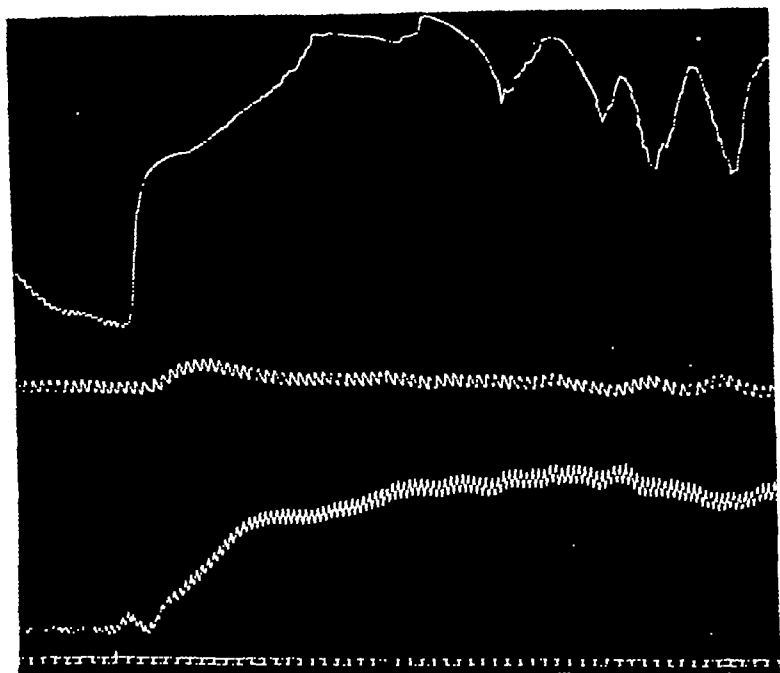


FIG 1

Cat Sb 38 Upper tracing Uterine contractions Middle tracing Perfusion pressure Lower tracing Maternal blood pressure Signal 1 unit pituitary (posterior lobe) extract injected intravenously into maternal circulation Time intervals 1 min

In addition to these variations in perfusion pressure it was also observed that the return of blood from the placenta into the reservoir of the perfusion pump was not a continuous flow, but was at a maximum during the period of uterine relaxation and during the early part of a contraction and then sharply decreased as the contraction developed. These results suggest that uterine tone and motility may play a part in aiding the return of blood from the placenta during the later stages of pregnancy. The results also offer evidence that the blood flow through the foetal placenta is markedly decreased by the contractions of the uterus.

Clark<sup>7</sup> found that during uterine contractions there occurred a transient rise followed by a fall in the foetal blood pressure and ascribed the fall to a reduced venous return from the placenta to the heart. This is in agreement with our own results on the return of blood from the placenta during uterine contractions.

<sup>7</sup> Clark, G. A. *J. Physiol.* 1932, 74, 391

## Effect of Metrazol on Cerebral Vessels

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At the beginning of a convulsive seizure in animals caused by metrazol (pentamethylenetetrazol), pallor of the brain surface has been reported. Liebert and Weil attribute this to "vasospasm".<sup>1</sup> Watterson and Macdonald, on the other hand, find "no certain evidence that the convulsion is either caused or accompanied by cerebral vasoconstriction" but they do find that drugs causing vasodilation tend to inhibit metrazol seizures.<sup>2</sup>

To get more accurate data on the vascular response to metrazol 15 cats and one monkey have been studied as follows. The diameters of pial arterioles and venules over the parietal cerebral cortex were measured by microscope through a cranial window.<sup>3</sup> Metrazol

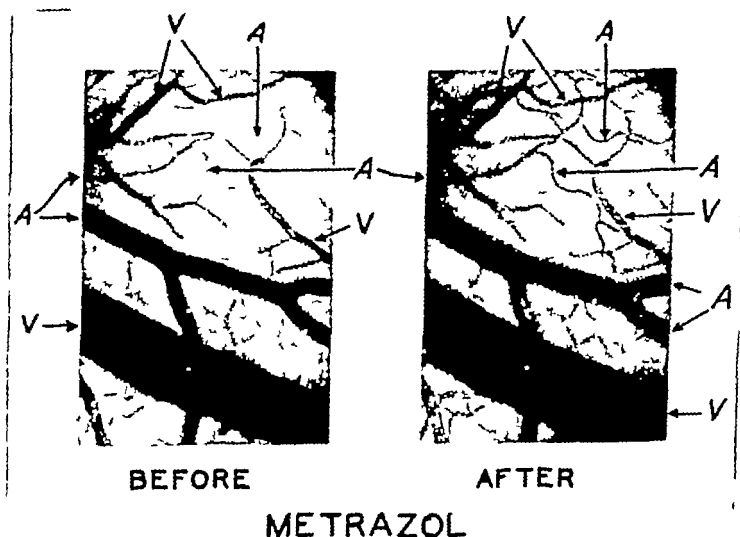


FIG 1

Photomicrographs of pial vessels before and after intravenous injection of 4% metrazol (40 mg per kilo). The arteries (A) veins (V) and their branches are all dilated in the second photo taken 65 seconds after the injection. Taken on 35 mm film—ocular  $\times 10$ , objective  $\times 3$ .

<sup>1</sup> Liebert, E, and Weil, A., *Arch Neurol and Psychiat*, 1939, 42, 690

<sup>2</sup> Watterson, D J, and Macdonald, R, *J Ment Sci*, 1939, 85, 392

<sup>3</sup> Forbes, H S, *Arch Neurol and Psychiat*, 1928, 19, 751

(8 to 80 mg per kilo, in 4% solution) was injected into the saphenous vein or carotid artery (46 trials), or it was applied locally to the surface of the brain (5 trials). Diallyl barbituric acid was used for anesthesia except in two instances, when intraperitoneal magnesium sulphate was used\*. Cerebrospinal fluid pressure from the cisterna magna was recorded by photokymograph<sup>4</sup>.

The findings were these. The pial arteries (and veins) dilated (Fig 1). The dilation usually started a few seconds before the first twitches were seen. Occasionally the seizure appeared first (Fig 2). After a 5-second intravenous injection of metrazol the latent period before the first sign of dilation averaged 10 seconds (26 trials). The latent period before the seizure averaged 15 seconds.

Sometimes, however, the vasodilation had passed and the vessels were contracting when the convulsion started (Fig 3) and some-

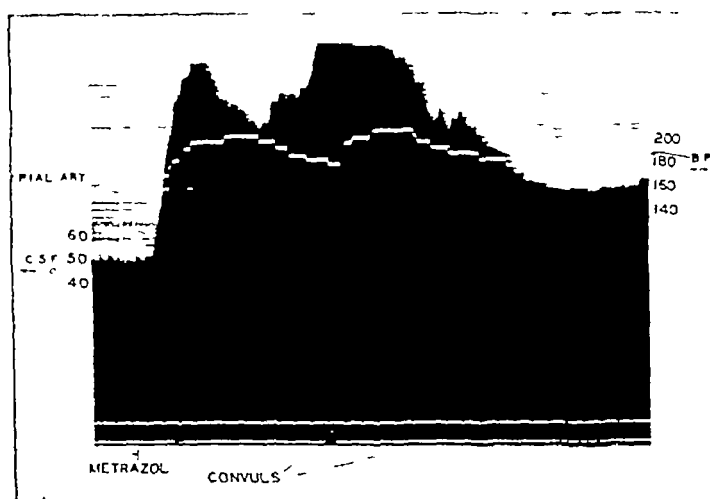


FIG 2

Dilation of pial artery. *Early convulsion.* Magnesium sulphate anesthesia. Artificial respiration. Metrazol—17 mg per kilo (1 cc 4% solution) was injected into a leg vein. Cerebrospinal fluid pressure (C.S.F.) is recorded by the top edge of the black area, which is the photographic tracing of the meniscus of fluid in a 1 mm bore manometer connected with the cisterna. Blood pressure was taken from the right common carotid. First convulsive twitches occurred one or 2 seconds before the pial artery started to dilate. No consistent relationship is apparent between C.S.F. pressure, blood pressure and arterial calibre.

\* The magnesium sulphate animals did not react to metrazol differently from the others except by showing often a rise in blood pressure.

<sup>4</sup> Forbes, H S, Nason G I, and Wortman, R C, *Arch Neurol and Psychiat*, 1937, 37, 334.

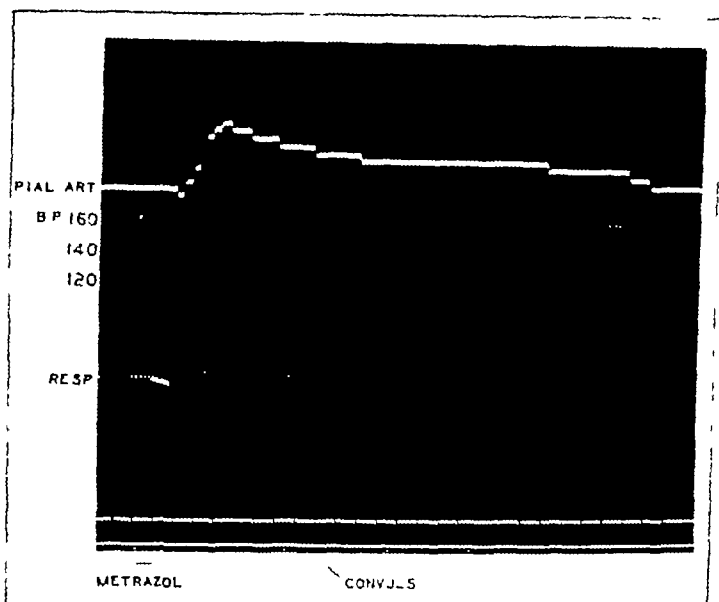


FIG 3

Dilation of pial artery Late convulsion Dial anesthesia Metrazol—40 mg per kilo (2 cc 4% solution) injected into a leg vein caused sudden fall in blood pressure Diameter of this pial artery was 254 microns Blood pressure was taken from the right common carotid Time marker records 5 second intervals Breaks in signal line (at bottom) indicate the presence of clonic muscular contractions The latent period before this seizure was 3 times longer than usual

times the dilation occurred without any convulsion (Fig 4) In other words, no constant relation was found between the seizure and the vascular change Vasoconstriction was not observed

The systemic arterial pressure after a period of fluctuation, usually fell Sometimes it remained almost unchanged Respirations often stopped for 5 or 10 seconds and then became very rapid In some experiments artificial respiration (with bilaterally opened thorax) was carried out Neither the incidence of convulsions nor of vasodilations was affected by changes in respiration or in blood pressure

Leibel and Hall found a decrease in cerebral blood flow (in rabbits) immediately following the injection of metrazol<sup>5</sup> They also noted a fall in blood pressure Undoubtedly this fall was the cause of the decreased flow, which took place in spite of the local vasodilation In those experiments of ours in which the blood pressure

<sup>5</sup> Leibel, B S, and Hall, G E, Proc Soc Exp Biol. and Med, 1938, 38, 894.



FIG 4

Dilation of pial artery. No convulsion. Dial anes. Lesia. Artificial respiration, open thorax. Metrazol—50 mg per kilo (3 cc 4% solution) injected in o/a leg vein caused a pronounced fall in blood pressure. The pial artery (104 microns in diameter) dilated promptly but no convulsion occurred. The rise in cerebrospinal fluid pressure—recorded by top of black area—happened in this instance to synchronize with the period of vasodilation. This relationship was not constant however.

remained constant or rose it is fair to assume that the flow increased.

The rise in cerebrospinal fluid pressure after each metrazol injection showed no constant relation to any of the other variables which were measured (Fig 2).

**Summary.** In animals metrazol caused cerebral vasodilation. This was unrelated to changes in blood pressure or in respiration, and showed no constant relation to convulsive seizures. No cerebral vasoconstriction was observed.

# Configuration of Glutamic Acid Isolated from Subacute Lymphatic Leukemic Tissue Proteins

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The exact relationship of leukemia to neoplastic disease has not been determined. The following types of human neoplasm, when examined by the procedure used by Kögl and Erxleben<sup>1</sup> have been reported to yield partly racemized glutamic acid<sup>2</sup>: carcinoma of the ovary, carcinoma of the breast, adenocarcinoma of the colon, and sarcoma of the thigh. Normal human tissues (ovaries, colon) have been reported to yield only 1(+)-glutamic acid<sup>2</sup>.

We have isolated glutamic acid from the liver and spleen of a patient who died of subacute lymphatic leukemia. Microscopic examination revealed that at least 20% of the liver, and 30% of the spleen, were composed of leukemic areas. Major portions of these organs removed at autopsy were placed in alcohol. Following transport to the laboratory, the tissues were finely minced, after which they were covered with 0.1 *N* hydrochloric acid and heated several hours on the steam bath until a majority of the material had gone into solution. Sufficient trichloroacetic acid then was added to make a concentration of 10%. The precipitated proteins were filtered off, washed and extracted several times with hot alcohol in order to remove trichloroacetic acid. The proteins were dried at 110° and lipids partly were extracted with boiling ether. Hydrolysis and isolation of glutamic acid were then carried out as described by Kögl, Erxleben, and Akkerman<sup>3</sup>. The results obtained are recorded in Table I. No racemic acid was isolated from the leukemic tissues.

TABLE I

	Melting point uncorrected	$[\alpha]_D^{25}$ * (in 9% HCl)	% of racemic glutamic acid
Leukemic liver	203-204°	+31.3°	0
" spleen	203-204°	+31.0°	0
Sarcoma (dog kidney)	201-202°	+27.8°	12

\* Literature +31.7°, calculated for free 1(+)-glutamic acid

<sup>1</sup> Kögl, F., and Erxleben, H., *Z. physiol. Chem.*, 1939, **258**, 57

<sup>2</sup> Kögl, F., *Z. Krebsforsch.*, 1939, **40**, 291; Arnow, L. E., and Opsahl, Jeanette C., *Science*, 1939, **90**, 257; White, J., and White, F. R., *J. Biol. Chem.*, 1939, **130**, 435

<sup>3</sup> Kögl, F., Erxleben, H., and Akkerman, A. M., *Z. physiol. Chem.*, 1939, **201**,

As a control, glutamic acid was isolated from protein obtained from a kidney neoplasm (dog). We are indebted to Dr E T Bell, Head of the Department of Pathology University of Minnesota Medical School, who examined sections of this tumor and found it to be an undifferentiated sarcoma

## 11331

### Configuration of Glutamic Acid Isolated from Proteins of Pig and Chick Embryo Tissues

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(Introduced by F H Scott)

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Minneapolis*

Since Kōgl and Erxleben<sup>1</sup> announced the isolation of partly racemized glutamic acid from malignant tissue protein, numerous confirmatory and non-confirmatory articles have appeared. The only laboratories other than Kōgl's that have investigated carcinoma protein by the Kōgl-Erxleben procedure have reported the isolation of partly racemized glutamic acid.<sup>2</sup> Dittmar<sup>3</sup> obtained only 1(+)glutamic acid from Rous sarcoma and from Jensen sarcoma free of necrosis, but later<sup>4</sup> found racemic glutamic acid in mouse sarcomas and carcinomas containing some necrotic material. Johnson<sup>5</sup> reported the isolation of glutamic acid containing small percentages of racemate from acid hydrolysates of Jensen sarcoma, but claimed that similar percentages were obtained also from normal mouse liver protein hydrolysates.

Kōgl, Erxleben and Akkerman<sup>6</sup> found only 1(+) glutamic acid in the hydrolysate of protein obtained from two- to three-months-old calf embryos. The embryonic tissues of other animals have not been investigated from this point of view. Since the metabolism of malignant tissue is similar in many respects to that of embryonic

<sup>1</sup> Kōgl F, and Erxleben, H, *Z physiol Chem* 1939, **258**, 57

<sup>2</sup> Arnow L. E., and Opsahl J. C., *Science* 1939, **90** 257. White J., and White, F. R. *J Biol Chem*, 1939, **130**, 435. Dittmar, C. *Z Krebsforsch.*, 1939, **49** 441

<sup>3</sup> Dittmar, C., *Z Krebsforsch.*, 1939, **49**, 397

<sup>4</sup> Dittmar, C., *Z Krebsforsch.* 1939, **49** 441

<sup>5</sup> Johnson I. M., *J Biol Chem* 1940, **132** 751

<sup>6</sup> Kōgl, F. Erxleben, H., and Akkerman A. M., *Z physiol Chem*, 1939, **261**,



tissue, obviously it is important to investigate the configuration of the glutamic acid obtained from the latter

We have isolated glutamic acid from the proteins of pig and chick embryo tissues. The isolations proved unexpectedly difficult, and glutamic acid hydrochloride crystallized only after standing in saturated hydrochloric acid solution for several weeks. Nine percent hydrochloric acid was used as a solvent in the determinations of optical rotation. The specific optical rotations reported in Table I have been calculated for free glutamic acid (literature  $+31.7^\circ$  for 1(+)-glutamic acid). The melting point (more properly, the decomposition point) of glutamic acid hydrochloride varies somewhat, depending on the method used for its determination. By the method employed by us, known pure 1(+)-glutamic acid hydrochloride melts at  $203\text{--}205^\circ$ .

TABLE I

	Protein, g	Glutamic acid HCl (Final yield) mg	Melting point	$[\alpha]_D$
Pig	9.2	37.4	$203\text{--}204^\circ$	$+31.8^\circ$
Chick	15.7	234.1	$203\text{--}204^\circ$	$+31.7^\circ$

*Experimental Pig Embryos* Pig embryos varying in length from 750 to 1000 mm, and in weight from 20 to 50 g each, were used. The bones were just beginning to calcify. Two hundred g of fresh total embryo tissue were macerated to a brei in a hand coffee mill. The brei was placed in a heavy cloth bag, and immersed several times in about 500 cc of boiling water to which a few drops of concentrated hydrochloric acid had been added. After centrifugation, the precipitated protein was washed with aqueous alcohol solution (1:4) until the washings were free of chloride. The washed protein was dried at  $110^\circ$ , pulverized in a mortar, and washed 3 times with boiling ether. Yield 9.2 g of protein. Hydrolysis and isolation of glutamic acid hydrochloride were carried out as described by Kogl, Erxleben, and Akkerman.<sup>6</sup>

*Chick Embryos* The entire bodies of 704 seven-day-old chick embryos were removed from eggs incubated by a standard method, blotted carefully with filter paper to remove excess moisture and weighed (397 g). After maceration, the tissue was covered with 0.1 N hydrochloric acid and heated on a water bath until a majority of the material had gone into solution (6-8 hours). Sufficient trichloroacetic acid was now added to make a concentration of 10% and the precipitated protein was allowed to settle. After filtration with suction, the protein was washed repeatedly with alcohol and ether.

(to remove trichloroacetic acid and the majority of adsorbed lipids) The protein thus isolated weighed 15.7 g following drying at 110°. Hydrolysis and isolation of glutamic acid hydrochloride again were carried out by the procedures used by Kogl, Erxleben, and Akkerman.<sup>6</sup>

*Summary* Proteins obtained from the embryonic tissues of chicks and pigs have been hydrolyzed, and the glutamic acid hydrochloride obtained from the hydrolysates examined in the polarimeter. The glutamic acid hydrochloride isolated was entirely the 1(+) variety.

### 11332 P

#### Effect of Trypsin on Development of *Rana pipiens*

ALFRED F. BLISS (Introduced by L. G. Barth)

*From the Department of Zoology, Columbia University*

Proteases secreted by the embryos of numerous species of fish and amphibia are believed to be important factors in bringing about hatching by digestion of the egg membranes. It has been asserted that in teleosts this protease is much like trypsin and so powerful that it attacks the embryo itself.<sup>1</sup> This statement conflicts with the dictum that living organisms are resistant to digestive enzymes. A striking confirmation of the latter view is Northrup's demonstration of the immunity of a wide variety of organisms to concentrated trypsin.<sup>2</sup>

These conflicting statements suggested the desirability of investigating the action of trypsin on the embryologically useful amphibian *Rana pipiens*. Since this anuran appears to possess a hatching enzyme the effect of trypsin on both membranes and embryonic viability was studied. The eggs were stripped of excess jelly on paper toweling and immersed in a 10% solution of dialyzed trypsin made up in 10% Ringer solution. Each experiment involved 25-50 eggs. Table I gives some typical results.

These results are in agreement with Northrup's work in their clear distinction between the susceptibility of the membrane proteins and the resistance of the living organism to trypsin. The possibility suggested by work on teleosts, of a general embryonic sensitivity to

<sup>1</sup> Needham, J., *Chemical Embryology* 1931, 3, 1597.

<sup>2</sup> Northrup, J. J. *Gen. Physiology* 1926, 9, 497.

tissue, obviously it is important to investigate the configuration of the glutamic acid obtained from the latter

We have isolated glutamic acid from the proteins of pig and chick embryo tissues. The isolations proved unexpectedly difficult, and glutamic acid hydrochloride crystallized only after standing in saturated hydrochloric acid solution for several weeks. Nine percent hydrochloric acid was used as a solvent in the determinations of optical rotation. The specific optical rotations reported in Table I have been calculated for free glutamic acid (literature  $+31.7^\circ$  for  $l(+)$  glutamic acid). The melting point (more properly, the decomposition point) of glutamic acid hydrochloride varies somewhat, depending on the method used for its determination. By the method employed by us, known pure  $l(+)$  glutamic acid hydrochloride melts at  $203-205^\circ$ .

TABLE I

	Protein, g	Glutamic acid HCl (Final yield) mg	Melting point	$[\alpha]_D$
Pig	9.2	37.4	$203-204^\circ$	$+31.8^\circ$
Chick	15.7	234.1	$203-204^\circ$	$+31.7^\circ$

*Experimental Pig Embryos* Pig embryos varying in length from 750 to 1000 mm, and in weight from 20 to 50 g each, were used. The bones were just beginning to calcify. Two hundred g of fresh total embryo tissue were macerated to a brei in a hand coffee mill. The brei was placed in a heavy cloth bag, and immersed several times in about 500 cc of boiling water to which a few drops of concentrated hydrochloric acid had been added. After centrifugation, the precipitated protein was washed with aqueous alcohol solution (1:4) until the washings were free of chloride. The washed protein was dried at  $110^\circ$ , pulverized in a mortar, and washed 3 times with boiling ether. Yield 9.2 g of protein. Hydrolysis and isolation of glutamic acid hydrochloride were carried out as described by Kogl, Erxleben, and Akkerman.<sup>6</sup>

*Chick Embryos* The entire bodies of 704 seven-day-old chick embryos were removed from eggs incubated by a standard method, blotted carefully with filter paper to remove excess moisture, and weighed (397 g). After maceration, the tissue was covered with 0.1 N hydrochloric acid and heated on a water bath until a majority of the material had gone into solution (6-8 hours). Sufficient trichloroacetic acid was now added to make a concentration of 10% and the precipitated protein was allowed to settle. After filtration with suction, the protein was washed repeatedly with alcohol and ether.

in mind, experiments were undertaken to determine whether the antibody, which accumulates in the papillomas in various amounts depending upon the titer of it in the blood and upon the local vascular conditions determining its extravasation may not influence the antigenicity of extracts of the growths

The antigenicity of the papilloma virus as determined by its capacity to elicit antibody upon intraperitoneal injection into normal rabbits was found to be markedly reduced when antibody was mixed with it *in vitro* in amounts sufficient to neutralize it. When an excess of antibody was added to a filtrate containing highly infectious virus, the mixture elicited no antibody upon repeated intraperitoneal injections into normal rabbits, although the control mixture containing saline and the same amount of virus (approximately 20 000 infectious doses for each animal) proved highly antigenic.

To procure virus-induced papillomas that were certain to contain the extravasated antibody in quantity a number of cottontail and domestic rabbits carrying vigorous confluent growths of 2 to 4 weeks' duration were injected intraperitoneally with large quantities of active virus. This greatly raised the titer of serum-antibody but resulted in no new lesions not only because the rabbits were already partially immune to the virus but because the virus acts only upon epidermis. After the serum-titer had remained for 10 days or more at the high level to which it had been brought the rabbits were killed for material. Although nourished by blood containing much antibody the growths had enlarged steadily—no unexpected finding since circulating antibody is known to be ineffective against virus associated with living papilloma cells\*. The growths were washed with soap and water and rinsed well to reduce the number of contaminating bacteria and then cut away with sterile instruments, diced and saved in 50% glycerol-Locke's solution in the refrigerator. Other rabbits not hyperimmunized but carrying growths of the same duration and derivation and hence having various but comparatively small amounts of antibody in their blood and presumably little extravasated antibody in the growths were likewise killed and their papillomas saved. After the growths had been kept in cold glycerol-Locke's for periods up to 2 weeks 1:10 or 1:20 saline suspensions were made of them. These were centrifugalized lightly and the supernatant liquids, all heavily opalescent but free from gross tissue-debris, were injected intraperitoneally into normal rabbits, the injections being repeated 8 or 9 days later. After a further

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\* Kidd, I. G., Beard, J. W., and Rous, P. *J. Exp. Med.* 1936, **64**, 63-70. Kidd, J. G. *J. Exp. Med.*, 1938, **67**, 551.

TABLE I

	Material	pH	Time, 20°C	Result
1	Eggs in early cleavage stages	ca 6	6 hr	Membranes fragile
2	" " " " "	" 8	3 6 "	Hatched
3	" " " " "	" "	1½ "	Membranes fragile
4	Membranes removed from 3	" "	6 "	Normal development
5	4, plus tr digestion residue	" "	12 "	Development stopped

digestive enzymes is not substantiated. While this may still be the case in teleosts, it seems more likely that the injurious action of the egg contents on the embryos was due to digestion products.

The parallelism between the effects of trypsin and normal hatching in *Rana pipiens* is in agreement with the theory that normal hatching is due to a digestive enzyme. It should be pointed out in this connection that the power of trypsin to mimic normal hatching makes it useful in large scale chemical work with early stages of anurans where the presence of the difficultly removable membranes may prove a hindrance to analysis or penetration.

## 11333 P

### Effect of Extravasated Antibody upon Antigenicity of Extracts of Virus-Induced Rabbit Papillomas

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Saline suspensions of virus-induced rabbit papillomas may stimulate the production of specific antiviral antibody when injected intraperitoneally into normal rabbits as Shope found, even though they contain no pathogenic virus demonstrable by the ordinary test<sup>1</sup>. In experiments of the same sort we found that saline extracts containing infectious papilloma virus in quantity elicited the antibody in much higher titer than extracts in which little or none was present<sup>2</sup>. Other studies already reported from this laboratory have shown that the antibody often extravasates into the large disorderly papillomas of cottontail rabbits in such quantity as to "mask" the causative virus<sup>3</sup> and that the antibody can be identified as such in extracts of the growths<sup>4</sup>. With these findings

<sup>1</sup> Shope, R. E., *J. Exp. Med.*, 1937, **65**, 219.

<sup>2</sup> Kidd, J. G., *Proc. Soc. Exp. Biol. and Med.*, 1938, **37**, 657, *J. Exp. Med.*, 1938, **68**, 703, 725, 737.

<sup>3</sup> Kidd, J. G., *J. Exp. Med.*, 1939, **70**, 583.

<sup>4</sup> Friedewald, W. F., *Proc. Soc. Exp. Biol. and Med.*, 1939, **42**, 380.

in mind, experiments were undertaken to determine whether the antibody, which accumulates in the papillomas in various amounts depending upon the titer of it in the blood and upon the local vascular conditions determining its extravasation may not influence the antigenicity of extracts of the growths

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\* Kidd, J. G., Beard, J. W. and Rous, P. *J. Exp. Med.* 1936 **64**, 63-70. Kidd, J. G., *J. Exp. Med.*, 1938 **67**, 551.

interval of 8 to 10 days the rabbits were bled from an ear vein and tested for resistance to the papilloma virus, with tests also of the serum for antibody by means of standardized neutralization and complement-fixation tests<sup>6</sup>

Materials derived from 10 wild and 11 domestic rabbits were used in 7 comprehensive experiments. The results were consistent and can be summarized together. Extracts of the 7 wild-rabbit papillomas that contained much infectious virus (and by inference little or no free antibody) invariably proved highly antigenic upon intraperitoneal injection into normal rabbits, eliciting antibody in amounts roughly proportional to the quantity of virus injected. Extracts of 3 wild and 6 domestic-rabbit papillomas, which contained in contrast little or no infectious virus demonstrable on test, elicited antibody in small or moderate amounts upon injection into normal rabbits. Extracts of the growths coming from 5 domestic rabbits having notably high serum-antibody titers failed completely to elicit antibody after repeated injections of large amounts (10 cc of 1:10 or 1:20 saline suspensions). The non-antigenic extracts invariably contained considerable amounts of extravasated antibody as was proved by their capacity to neutralize added virus *in vitro*. The fact was noted incidentally that passively transferred antibody is often responsible for the resistance to the virus that becomes manifest after extracts of papillomas containing much extravasated antibody are injected intraperitoneally into normal rabbits.

The findings would appear to warrant the conclusion that antibody can reduce or abolish the antigenicity of the papilloma virus upon mixture with it *in vitro* and that sufficient antibody can extravasate into the virus-induced papillomas of wild and domestic rabbits not only to neutralize the virus liberated when the growths are extracted but also to render extracts or suspensions of some growths incapable of eliciting antibody upon injection into normal rabbits. The findings disclose the limitations of immunization-experiments of the sort outlined as a means of demonstrating "masked" virus. Since much antibody is present in the blood of cottontails having cancers deriving naturally from the virus-induced papillomas and since extravasated antibody has been demonstrated in extracts of the cancers,<sup>7</sup> it follows that the attempted immunization of normal rabbits with extracts of these growths will fail to provide decisive evidence as to whether the virus is or is not present in the malignant tissue, unless the effects of extravasated antibody can first be excluded.

<sup>6</sup> Kidd, J. G., Beard, J. W., and Rous, P., *J. Exp. Med.* 1936, **64**, 63-79; Kidd, J. G., *J. Exp. Med.*, 1938, **68**, 703, 725, 737.

<sup>7</sup> Kidd, J. G., and Rous, P., *J. Exp. Med.*, 1940, **71**, 469.

## Demonstration of a Capsule on *Brucella* Cells

I FOREST HUDDLESON (Introduced by W L Mallman )

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All smooth strains of the 3 species of *Brucella* have been found to possess a definite capsule.

The technic for demonstrating the presence of a capsule on *Brucella* is as follows. A dilute suspension of organisms is prepared in physiological salt solution from a 48-hour liver agar slant growth. A drop of the suspension is drawn across a clean glass slide similar to the method of making a blood film. Allow the film to dry in air. Place a small drop of Kesso India ink near one end of the slide and draw it over the film of organisms by placing the end edge of another slide at such an angle that the India ink spread becomes thinned out. After air drying, the spread is flooded with an aqueous solution of crystal violet (5 cc of saturated alcoholic solution of crystal violet in 95 cc distilled water). The excess of stain is washed off the slide with distilled water at the end of 2 minutes. The stained spreads are dried in air.

When examined under the microscope (magnification 1000 $\times$ ), the organisms appear as dark purple cocci or rods located in the center or near the edge of a clear area surrounded by India ink. The clear area which is the capsule varies in size from 2.5 to 4. The size of the capsule varies with the size of the stained cell.

The writer has met with no success in staining the capsule on *Brucella* cells.

The rough form of *Brucella* also shows a capsule. A large proportion of the cells of this form emerge from the capsule when suspended and allowed to stand in distilled water at 37°C. As the cell leaves the capsule, the cell becomes swollen and more intensely stained.

The capsule may be removed from the cell by digestion with hot chloroform-ether mixture acidified with HCl. This treatment also destroys that part of the organism which is made visible by the usual staining methods.

Preliminary studies now in progress in this laboratory show that the capsular material is composed of lipids in close combination with a polysaccharide.

Capsular swelling has not been demonstrated when organisms are incubated with specific serum.



interval of 8 to 10 days the rabbits were bled from an ear vein and tested for resistance to the papilloma virus, with tests also of the serum for antibody by means of standardized neutralization and complement-fixation tests<sup>6</sup>

Materials derived from 10 wild and 11 domestic rabbits were used in 7 comprehensive experiments. The results were consistent and can be summarized together. Extracts of the 7 wild-rabbit papillomas that contained much infectious virus (and by inference little or no free antibody) invariably proved highly antigenic upon intraperitoneal injection into normal rabbits, eliciting antibody in amounts roughly proportional to the quantity of virus injected. Extracts of 3 wild and 6 domestic-rabbit papillomas, which contained in contrast little or no infectious virus demonstrable on test, elicited antibody in small or moderate amounts upon injection into normal rabbits. Extracts of the growths coming from 5 domestic rabbits having notably high serum-antibody titers failed completely to elicit antibody after repeated injections of large amounts (10 cc of 1:10 or 1:20 saline suspensions). The non-antigenic extracts invariably contained considerable amounts of extravasated antibody, as was proved by their capacity to neutralize added virus *in vitro*. The fact was noted incidentally that passively transferred antibody is often responsible for the resistance to the virus that becomes manifest after extracts of papillomas containing much extravasated antibody are injected intraperitoneally into normal rabbits.

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<sup>6</sup> Kidd, J. G., Beard, J. W., and Rous, P., *J. Exp. Med.* 1936, **64**, 63-79; Kidd, J. G., *J. Exp. Med.*, 1938, **68**, 703, 725, 737.

<sup>7</sup> Kidd, J. G., and Rous, P., *J. Exp. Med.*, 1940, **71**, 469.

fanilamidothiazol) and sulfamethylthiazol (2-sulfanilamidomethylthiazol) were added to this culture medium. The drugs were dissolved by heating the broths in a water bath. Then, the broths were tubed and autoclaved at 15 lb pressure for 12 minutes. It may be mentioned that a change of the pH to the acid side occurred when sulfanilyl-sulfanilamide, sulfthiazol and sulfamethylthiazol were dissolved in the broth.

The strains of hemolytic and non-hemolytic enterococci were the same as those previously used in a study on the bacteriostatic action of sulfanilamide upon members of the enterococcus group<sup>5</sup>. In addition to these, several strains were obtained through the courtesy of Dr. H. F. Helmholz, the Mayo Clinic, Rochester, Minnesota. The strains were cultured in brain-heart infusion broth for 18-24 hours, this culture medium was also used for diluting purposes.

Table I presents an experiment in which the bacteriostatic action of various sulfanilamide derivatives in a concentration of 100 mg % upon a strain of hemolytic enterococcus (dilution of 1:125,000) was tested both at 37°C and 43°C. It may be seen from this table, that (1) at 37°C, sulfanilamide and sulfapyridine lacked growth inhibitory action, whereas sulfanilyl-sulfanilamide and sulfathiazol delayed the growth of the microorganisms for a short period only. (2) At 43°C, sulfanilamide failed to inhibit the growth of the enterococcus, sulfapyridine delayed its growth, and sulfanilyl-sulfanila-

TABLE I  
Bacteriostatic Action of 100 mg % of Sulfanilamide, Sulfapyridine, Sulfanilyl sulfanilamide and Sulfathiazol upon a Strain of *Enterococcus hemolyticus* in  $\frac{1}{4}$  % Maltose Phenol Red Broth

	1	2	3	4	5
Hr of Incubation	Control broth	Sulfanilamide broth	Sulfapyridine broth	Sulfanilyl sulfanilamide broth	Sulfathiazol broth
Incubation at 37°C					
1 10	++	+	++	—	—
2 18	++++	++++	++++	+++	++++
3 24	++++	++++	++++	++++	++++
4 48	++++	++++	++++	++++	++++
5 72	++++	++++	++++	++++	++++
6 120	++++	++++	++++	++++	++++
Incubation at 43°C					
1 10	—	—	—	—	—
2 18	+++	+++	—	—	—
3 24	+++	+++	+++	—	—
4 48	+++	+++	+++	—	—
5 72	+++	+++	+++	—	—
6 120	+++	+++	+++	—	—

— = No visible growth

+ to ++++ = various degrees of growth

<sup>5</sup> Neter, E, PROC SOC EXP BIOL AND MED, 1940, 43, 52

# Comparative Study on Bacteriostatic Action of Sulfanilamide, Sulfapyridine, Sulfanilyl-Sulfanilamide and Sulfathiazol upon Enterococci

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At the present time it is not as yet possible to predict from the chemical composition of various sulfanilamide derivatives their chemotherapeutic activity toward different microorganisms that are more or less susceptible to the action of sulfanilamide. Thus, it is necessary to test the efficacy of new compounds both *in vitro* and *in vivo*. It is generally agreed that sulfanilamide in therapeutic doses has little or no effect on enterococci. Recently Helmholz<sup>1</sup> reported that sulfathiazol and sulfamethylthiazol in urine are bactericidal toward enterococci. Hill<sup>2</sup> found that sulfanilamide, sulfapyridine and sulfathiazol in a concentration of 100 mg % have a marked bacteriostatic or germicidal action upon relatively small numbers of *alpha-Streptococcus fecalis* in urine. This author made the important observation that sulfathiazol as present in urine after administration of the drug exerts greater bacteriostatic activity than the same amount added to normal voided urine. Long and Bliss<sup>3</sup> observed that sulfanilamide, sulfapyridine and sulfathiazol in concentrations of 10 mg % only slightly inhibited the growth of hemolytic streptococcus zymogenes (Group D) in beef infusion, 2% Neopeptone, 0.075% dextrose broth. Clinically, Pool and Cook<sup>4</sup> reported sterilization of urine of patients harboring *Streptococcus fecalis* following the administration of sulfathiazol and sulfamethylthiazol, respectively. The concentration of the drug in the urine of these cases exceeded 150 mg %. The following *in vitro* experiments were carried out in order to determine the relative bacteriostatic activity of various sulfanilamide derivatives toward hemolytic and non-hemolytic enterococci.

As culture medium  $\frac{1}{4}$  % maltose phenol red broth base (Difco), containing tryptose (1%), sodium chloride (0.5%), dipotassium phosphate (0.1%), and phenol red, was used. Sulfanilamide, sulfapyridine, sulfanilyl-sulfanilamide (Disulon), sulfathiazol (2-sul-

<sup>1</sup> Helmholz, H. F., *Proc Staff Meetings Mayo Clinic*, 1940, **15**, 65.

<sup>2</sup> Hill, J. H., *J Urol*, 1940, **43**, 491.

<sup>3</sup> Long, P. H., and Bliss, E. A., *Proc Soc Exp Biol. and Med.*, 1940, **43**, 324.

<sup>4</sup> Pool, T. L., and Cook, E. N., *Proc Staff Meetings Mayo Clinic*, 1940, **15**, 113.

## 11336 P

Acetylmethylcarbinol Enzyme-System of *Aerobacter aërogenes*

M SILVERMAN AND C H WERKMAN

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It is generally accepted that in the formation of acetylmethylcarbinol from pyruvic acid by such bacteria as *Aerobacter aërogenes*, the acid must be first decarboxylated to acetaldehyde. No direct evidence, however, has been presented that *Aerobacter* can bring about such a decarboxylation. The difficulty has been due, in part, to the simultaneous activity of hydroclastic enzymes, *i. e.*, enzymes splitting pyruvic acid into formic and acetic acids (*cf.* equation 1). The formic acid is cleaved into  $H_2$  and  $CO_2$ . The formation of  $CO_2$  by this reaction does not involve intermediary acetaldehyde.

Employing the technic of Wiggert *et al.*,<sup>1</sup> an enzyme-preparation has been extracted from cells of *Aerobacter aerogenes*, with apparently no hydroclastic activity, nevertheless, capable of converting pyruvic acid into  $CO_2$  and acetylmethylcarbinol. Typical fermentation-balances are shown in Table I. The ratio of pyruvate utilized to  $CO_2$  and acetylmethylcarbinol formed is approximately 2.2:1.

Attempts to increase the carbinol yield by the addition of acetaldehyde were unsuccessful. The added aldehyde was recovered unchanged. If acetaldehyde is an intermediate, only the biologically nascent form is utilized by this enzyme-preparation.

As indicated in Table II, no appreciable production of  $CO_2$  and consequently no carbinol occurs at pH levels above 6.8. There is a direct relationship between  $CO_2$  and carbinol production.

The relationship between pH and the direct cleavage of  $CO_2$  from

TABLE I  
Dissimilation of Pyruvic Acid by Enzyme-preparation of *A. aërogenes*  
Values in millimoles

Initial pyruvic acid	Final pyruvic acid	Pyruvic acid utilized	$CO_2$	Acetylmethyl carbinol
9.17	1.60	7.57	7.63	3.30
9.17	1.69	7.48	7.42	3.40
4.99	1.08	3.91	3.62	1.61
2.49	0.16	2.33	—	1.09

3 ml enzyme preparation vol. 30 ml 5 ml M/15  $KH_2PO_4$  atmosphere  $N_2$  30°C

<sup>1</sup> Wiggert, W. P., Silverman, M., Utter, M. F., and Werkman, C. H. *Iowa State Coll. J. Sci.* 1940, 14, 179.

mide and sulfathiazol prevented visible growth for 5 days (3) The growth of the enterococcus in the control broth was slightly retarded and inhibited at 43°C in comparison to that obtained at 37°C

As in the case of sulfanilamide, the degree of bacteriostasis exerted by sulfanilyl-sulfanilamide and sulfathiazol depends, besides on other factors, upon the size of the inoculum, *c g*, in one particular experiment sulfathiazol in a concentration of 0.1% did not delay the growth of a hemolytic enterococcus when 0.1 cc of a 1:50 diluted broth culture was used for inoculation, with a 1:2500 dilution it delayed the growth for 18 hours and with a 1:6,000,000 dilution it completely prevented visible growth for 8 days. Essentially the same results were obtained with hemolytic and non-hemolytic enterococci.

In preliminary experiments with sulfamethylthiazol it was found that this drug in concentrations of 0.1% or in saturated solution is bacteriostatic toward members of the enterococcus group and is more effective than sulfanilamide in equal concentrations (0.1% to 0.2%)

Previously, it was shown\* that sulfanilamide in concentration of 1% may completely prevent visible growth of suitable numbers of enterococci in broth incubated at approximately 43°C. In order to further evaluate the relative efficacy of sulfanilamide and its derivatives, the bacteriostatic effect of 1% sulfanilamide was compared with that of sulfanilyl-sulfanilamide, sulfathiazol and sulfamethylthiazol in 0.2% concentration or saturated solution, respectively.

These experiments were carried out with hemolytic and non-hemolytic enterococci. It was found that 1% sulfanilamide is of greater effectiveness than are sulfanilyl-sulfanilamide, sulfathiazol and sulfamethylthiazol in the above mentioned concentrations.

In conclusion (1) Sulfanilyl-sulfanilamide, sulfathiazol and sulfamethylthiazol in concentrations of 100 mg % or above exert definite bacteriostatic activity toward small numbers of both hemolytic and non-hemolytic enterococci in 1/4% maltose broth at 43°C. (2) The growth inhibitory effect of these drugs is greater than that of equal concentrations (0.1% to 0.2%) of sulfanilamide and sulfapyridine. (3) The bacteriostatic effect of sulfanilamide and its derivatives upon the growth of enterococci is greater at 43°C than at 37°C.

We wish to express our appreciation to Alba Pharmaceutical Company for the supply of sulfanilyl-sulfanilamide (Disulon), to Merck & Company for sulfapyridine, to E. R. Squibb & Sons for sulfathiazol and to Winthrop Chemical Company for sulfamethylthiazol.

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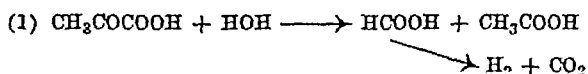
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TABLE II  
Relationship Between pH and Rate of CO<sub>2</sub> Formation from Pyruvic Acid by  
Enzyme-preparation of *A. aerogenes*

pH	3.5	5.6	6.0	6.4	6.8	7.2	7.6	8.0
mm <sup>3</sup> CO <sub>2</sub> in 15 min	170	122	119	76	25	11	4	9
V P test	4+	4+	4+	4+	2+	?	—	—

15 ml juice vol 2.0 ml 1.8 mg pyruvic acid 1 ml PO<sub>4</sub> buffer M/15 atmosphere air

pyruvic acid by *Aerobacter* will assist in elucidating the mechanism of pyruvate-breakdown. From the investigations of Mickelson and Werkman<sup>2</sup> and from Table II, the carbinol is not formed at alkaline reactions and the predominating mechanism involves reaction I. Under acid conditions both reactions occur. Mickelson<sup>3</sup> has shown that the products of the alkaline fermentation of pyruvate by *Aerobacter* are acetic and formic acids in equimolar proportions.



These results may be checked manometrically with cell suspensions as shown in Table III. As the system is made alkaline, more H<sub>2</sub> should be evolved as reaction 2 becomes inhibited. At pH 8.0 the CO<sub>2</sub>/H<sub>2</sub> ratio closely approaches the theoretical value of 1.0.

The presence of inorganic phosphate has been found to be essential for pyruvate-breakdown by this enzyme-preparation.

TABLE III  
pH and the Gas Ratios of *A. aerogenes* (whole cells) on pyruvate

pH	4.5	5.6	6.8	8.0
mm <sup>3</sup> H <sub>2</sub>	164	208	335	370
mm <sup>3</sup> CO <sub>2</sub>	426	449	445	398
CO <sub>2</sub> /H <sub>2</sub>	2.60	2.16	1.33	1.08

Vol 2.3 ml atmosphere N<sub>2</sub>-cell 24 hours old 2.2 mg pyruvic acid.

<sup>2</sup> Mickelson, Milo, and Werkman, C. H., *J. Bact.*, 1938, **36**, 67.

<sup>3</sup> Mickelson, Milo, unpublished thesis, Iowa State College, 1939.

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7531 P

Injections of Sodium Fluoride on Enamel and Dentin of the  
Incisor of the Rat.\*

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*From the Department of Histology, University of Illinois, College of Dentistry,  
and Department of Nutrition, Agricultural Experiment Station, University of  
Arizona.*

The microscopic anatomy of the incisors was studied in 3 groups of rats

A 12 rats that were given 2 to 8 injections of 3 cc of 2.5% sodium fluoride 24 or 48 hours apart Age 90-270 days

B 20 rats that were given single injections of 3 cc of 2.5% sodium fluoride and allowed to live 1 to 48 hours after the administration Age 90-270 days

C 16 controls Most of these were littermate controls

Significant alterations were observed only in Groups A and B. The histologic changes in group A were

1 Both the enamel and dentin show a pair of light (disturbed) and dark (recovery) incremental layers for each injection of so-

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\* Technical bulletin No. 52 of the University of Arizona Agricultural Station gives a more complete description of some of the phases of this report

dium fluoride The width of each pair is approximately  $32\mu$  for injections given 48 hours apart and approximately  $16\mu$  for injections given 24 hours apart

2 The light layers represent the immediate response to the injections and are imperfect in formation and calcification

3 The dark layers represent a recovery response and are normal in formation and normal or excessive in calcification

4 This incremental pattern is a constant finding but shows disturbances when the administration is continued for more than 5 injections at 24-hour intervals

The histologic changes in group B varied with the time interval between the injection and death

1 48-hour interval One pair of light and dark incremental layers in the enamel and dentin

2 12 to 24-hour interval a the incremental surface of the organic enamel matrix lacks its normal arrangement and is covered with hemispherical globules that stain deeply with hematoxylin, b an abnormal character and distribution of globules within the ganoblastic layer situated in the posterior portion of the incisor

3 1-6-hour intervals Abnormal character and distribution of globules within the ganoblastic layer of the posterior and formative portion of the incisor

The injection of fluorine offers an accurate and easy method of measuring the rate of growth of the enamel and dentin in continuously growing teeth

It is believed that fluorine exerts a direct local action on the enamel-forming cells and that the changes observed in the enamel and dentin are not produced primarily by changes in blood calcium and phosphorus The nature of the cytologic disturbances is being investigated further

## 7532 P

## Bleeding Volume in Experimental Burns

HENRY N HARKINS (Introduced by Edmund Andrews)

*From the Douglas Smith Foundation and the Department of Surgery, University of Chicago*

Roome, Keith, and Phemister<sup>1</sup> showed that in experimental shock due to hyperventilation, anaphylaxis, histamine administration, spinal cord section, and spinal anesthesia the bleeding volume averaged 49.9% of the calculated blood volume (one-thirteenth of the body weight). This is only slightly less than the average of 58.6% obtained for control dogs. On the other hand, in shock due to trauma to an extremity, hemorrhage, plasmapheresis, and intestinal manipulation, the bleeding volume was greatly reduced, averaging 21.8%. Blalock<sup>2</sup> showed that the cardiac output in shock due to severe burns is markedly reduced. The work reported in the present paper was done to determine the bleeding volume in shock due to experimental burns.

Dogs under complete barbitol anesthesia and suffering no pain were used throughout the work. They were burned and at intervals the carotid blood pressure, hematocrit reading, and hemoglobin percentage were determined. When the blood pressure had fallen to about 80 mm. of mercury the animal was bled to death through a large carotid cannula. The time interval between the burning and the bleeding varied from 16 to 24 hours in 7 dogs. The hematocrit reading and hemoglobin percentage rose steadily from the time of burning to the time of bleeding. On the other hand, the blood

TABLE I  
Bleeding Volumes of Dogs with Experimental Burns

No	Wt., Kg	Interval from burning to bleeding	Blood pressure mm. Hg		Terminal bleeding vol. % cal. blood vol.
			Start	End	
		hr min			
1	10.4	17 40	100	52	11.9
2	9.2	16 15	148	44	14.6
3	11.3	17 50	154	108	29.3
4	7.8	23 10	116	48	16.1
5	10.5	21 40	162	66	16.7
6	12.1	23 45	148	86	28.4
7	6.8	18 20	118	66	24.9
Aver					20.3

<sup>1</sup> Boome, N. W., Keith, W. S., and Phemister, D. B., *Surg. Gynec. and Obstet.*, 1933, **56**, 161.

<sup>2</sup> Blalock, A. *Proc. Soc. Exp. Biol. and Med.*, 1933, **31**, 36.



dium fluoride The width of each pair is approximately  $32\mu$  for injections given 48 hours apart and approximately  $16\mu$  for injections given 24 hours apart

2 The light layers represent the immediate response to the injections and are imperfect in formation and calcification

3 The dark layers represent a recovery response and are normal in formation and normal or excessive in calcification

4 This incremental pattern is a constant finding but shows disturbances when the administration is continued for more than 5 injections at 24-hour intervals

The histologic changes in group B varied with the time interval between the injection and death

1 48-hour interval One pair of light and dark incremental layers in the enamel and dentin

2 12 to 24-hour interval a the incremental surface of the organic enamel matrix lacks its normal arrangement and is covered with hemispherical globules that stain deeply with hematoxylin, b an abnormal character and distribution of globules within the ganoblastic layer situated in the posterior portion of the incisor

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The injection of fluorine offers an accurate and easy method of measuring the rate of growth of the enamel and dentin in continuously growing teeth

It is believed that fluorine exerts a direct local action on the enamel-forming cells and that the changes observed in the enamel and dentin are not produced primarily by changes in blood calcium and phosphorus The nature of the cytologic disturbances is being investigated further

## 7533 P

## Toxicity of Heavy Water \*

FREDERICK E KREDEL, HENRY N HARKINS AND WILLIAM D  
HARKINS (Introduced by E Andrews)

*From the Departments of Surgery and Chemistry of The University of Chicago*

The following experiments were performed in an effort to determine whether or not the newly recognized substance, heavy water (deuterium oxide) possesses toxic properties. Water containing approximately 20% deuterium oxide was prepared electrolytically and its action tested on rabbit leucocytes and bacteria. Experiments are now being done testing heavy water in more concentrated form up to 100%.

*Experiment 1—Rabbit leucocytes* One drop of a 24-hour aleurinate broth exudate, freshly removed from the pleural cavity of a rabbit, was added to 2 cc. of a modified Tyrode solution containing 18% heavy water and 1/10,000 neutral red. The Tyrode solution, which was buffered at pH 6.6, had 10% of its salts replaced by  $\text{NH}_4\text{Cl}$ . Two hanging drops and one spread preparation, sealed with vaseline-paraffin mixture, were incubated at 37°C and observed under the microscope. A control series was prepared with triple distilled water.

The viability of the leucocytes was followed. After one hour an occasional dead cell could be seen. In 8 hours 10% were dead, in 16 hours 50%, and in 24 hours about 75% were dead. There were no significant differences between leucocytes in the heavy water solution and control ordinary water preparations.

*Experiment 2—Growth of Staphylococcus* A minute amount of an 18-hour broth culture of hemolytic *Staphylococcus aureus* was added to a drop of 20% heavy water. The drop was divided into 3 parts which were sealed on sterile cover slip hanging drops. These were cultured on blood agar plates after 6, 24 and 48 hours respectively. There was no inhibition of growth.

B A drop of 20% heavy water was placed in the center of a blood agar plate freshly streaked with the organism used in A. No effect was noted during a period of 4 days.

*Experiment 3—Motility of B Typhosus* A shaken loop of an 18-hour ascitic-broth culture of *B typhosus* was stirred into 2 hang-

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\* Work done in part under a grant from the Douglas Smith Foundation

pressure usually remained near the normal level until the end of the experiment and then fell more rapidly. The average bleeding volume in the 7 burned dogs was 20.3% of the calculated blood volume (one-thirteenth of the body weight) as shown in Table I. This figure agrees quite well with that of 21.8% which Roome, Keith, and Phemister found in similar types of shock. The extent of the burn in these dogs may be gauged as follows. After death the dogs were bisected according to the method of Blalock<sup>3</sup> and the weight of the normal and burned sides of the animal compared. This difference was 3.0, 2.5, 2.2, 0.3, 1.1, 3.0, and 2.4, averaging 2.1% of the total body weight in the 7 dogs and represents a fluid loss into the tissues on the burned side sufficient to account for the shock present in these animals.

In 4 control dogs that were similarly treated except that they were not burned, the average bleeding volume was 53.4% of the calculated blood volume as shown in Table II. This figure agrees

TABLE II  
Bleeding Volumes of Control Dogs

No	Wt. Kg	Interval from start exper to bleeding	Blood pressure mm Hg		Terminal bleeding vol. % cal. blood vol.
			Start	End	
		hr min.			
1	24.0	24 0	154	128	49.8
2	13.0	17 20	134	136	52.0
3	12.1	15 40	136	102	47.2
4	19.5	18 20	118	116	64.7
Aver					53.4

rather well with that of 58.6% which Roome, Keith, and Phemister found for control dogs. Any slight discrepancy may be explained by the fact that their dogs were bled one hour after beginning the experiment, whereas in the present paper the time interval in the control series was from 16 to 24 hours.

The blood pressure, hematocrit reading and hemoglobin percentage fell very slightly during this interval.

From a comparison of the results in the burned and control series it is concluded that the bleeding volume is markedly reduced in experimental burns.

<sup>3</sup> Blalock, A. *Arch. Surg.*, 1931, **22**, 610

tract Bile peritonitis was induced in 9 dogs by 3 methods by the intraperitoneal injection of whole sterile dogs' bile, by the similar administration of a sterile bile salt solution and by ligature of the common bile duct followed by defundation of the gall bladder Twenty-three specimens of peritoneal washings or of peritoneal exudates were obtained by aseptic lavage of animals with suppurative peritonitis some time prior to death These fluids were then centrifuged at high speed until clear and the supernatant fluid injected intravenously into normal dogs under barbital or urethane anesthesia In a few instances the supernatant fluid was passed through a Mandler filter of porosity corresponding to a Berkefeld-N filter Since the effect on blood pressure of the Mandler filtrates differed only quantitatively from the centrifuged supernatant fluid, the 2 will be considered collectively As a control 18 fluids were obtained by aseptic lavage of the peritoneal cavity of 13 normal dogs Extracts were made by the method of Chang and Gaddum<sup>5</sup> of the whole peritoneal washings, the supernatant fluids, and of the centrifuged sediment from all the above materials

The symptoms described by the investigators<sup>1-3</sup> who demonstrated the presence of the soluble toxic substance of *Escherichia coli* are compatible with those produced by a profound drop in blood pressure Accordingly, we have also investigated the hemodynamic effects of bacterial filtrates of broth cultures of *Escherichia coli* and of a hemolytic streptococcus freshly isolated from a case of streptococcus peritonitis in man Extracts by the method of Chang and Gaddum<sup>5</sup> were also prepared of the corresponding bacterial sediments in several instances Peptone-free veal infusion broth was used for the nutrient medium Aerobic and anaerobic cultures were made in every instance when the peritoneal cavity was opened In those cases where a significant drop in blood pressure was obtained upon injection of the peritoneal washings into a normal dog there was a heavy growth of *Escherichia coli* with or without a growth of an obligate anerobe resembling *Clostridium welchii*

Results (a) *Peritonitis induced by an open intestinal loop* In 15 instances taken from tests of the 23 specimens of peritoneal washings from dogs with peritonitis produced by an open intestinal loop, there was a profound but variable immediate drop in blood pressure of from 18 to 80 mm of mercury Three specimens obtained post-mortem varied from the others in that the latter samples were tainted by the odor of putrefaction These latter 3 gave an especially rapid and profound drop in blood pressure A considera-

<sup>5</sup> Chang, H C, and Gaddum, J H, *J Physiol*, 1933, 79, 255

ing drop preparations of heavy water and into 2 controls with distilled water. Over a period of 48 hours no difference in motility could be observed between the heavy water and control ordinary water preparation.

## 7534 P

## Depressor Substances in Peritonitis

PAUL H. HARMON AND HENRY N. HARKINS (Introduced by Edmund Andrews)

*From the Department of Surgery, The University of Chicago \**

There is little experimental work to substantiate the clinical belief that death from peritonitis is due to vasomotor collapse incident to absorption of toxins from the peritoneum. Zinnser, Parker and Kuttner<sup>1</sup> and Branham<sup>2</sup> both demonstrated that *Escherichia coli* produced a soluble toxic substance. Steinberg and Ecker<sup>3</sup> and Steinberg<sup>4</sup> have emphasized the rôle of bacterial toxins in peritonitis. Steinberg and his co-workers<sup>5</sup> have demonstrated a slight blood pressure fall in early peritonitis. Scott and Wangensteen<sup>6</sup> have showed that the peritoneal exudates from uncomplicated experimental intestinal obstruction were innocuous.

It occurred to us that the vasomotor system of the host might be less sensitive than that of a normal animal to the toxic substances developed in the peritoneal cavity. Peritonitis was induced in 17 dogs by the method of Buchbinder, Heilman and Foster<sup>7</sup> which consists of leaving an open loop of ileum with intact blood supply free in the peritoneal cavity. An end to end or a lateral anastomosis is made around the loop to restore the continuity of the intestinal

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\* Supported in part by a grant from the Douglas Smith Foundation for Medical Research of the University of Chicago.

<sup>1</sup> Zinnser, H., Parker, J. T., and Kuttner, A., *Proc. Soc. Exp. Biol. and Med.*, 1920, **18**, 49.

<sup>2</sup> Branham, S. E., *J. Infect. Dis.*, 1925, **37**, 533.

<sup>3</sup> Steinberg, B., and Ecker, E. E., *J. Exp. Med.*, 1926, **43**, 443.

<sup>4</sup> Steinberg, B., *Arch. Surg.*, 1931, **23**, 145.

<sup>5</sup> Steinberg, B., Kobacker, J. L., and Russel, T. G., *Proc. Soc. Exp. Biol. and Med.*, 1930, **30**, 1155.

<sup>6</sup> Scott, H. G., and Wangensteen, O. H., *Proc. Soc. Exp. Biol. and Med.*, 1932, **20**, 559.

<sup>7</sup> Buchbinder, J. B., Heilman, F. R., and Foster, G. C., *Surg. Gyn. and Obst.*, 1931, **53**, 726.

instance contains 90-120 mg of dextrose per 100 cc of blood. The presence of the colloids inhibits even *in vitro* an increase of this amount within certain (physiological) limits. If we try to increase the blood sugar by adding the physiological amount of 100 mg per 100 cc and determine the sugar we find only 25% of the added amount, 75% (60-80%) disappear in the normal human serum. The serum colloids have, therefore, the power to restore and to maintain the equilibrium, if we try to disturb it. dynamic equilibrium. The following studies are based on this observation. The serum crystalloids are electrolytes and non-electrolytes. The influence of changes of the electrolytes as well as of the non-electrolytes on the static and on the dynamic equilibrium of the non-electrolytes of the normal human blood serum has been studied with the following results.

(1) Influence of cations on the static equilibrium of the serum sugar. Chlorides have been used in n/10, n/100 solution, 0.02 cc added to 1 cc of serum. NaCl does not influence the blood sugar even in n-solutions. KCl increases the blood sugar (5-10%),  $\text{CaCl}_2$  and  $\text{MgCl}_2$  do not influence the sugar as a rule, but act differently in different sera.  $\text{FeCl}_3$  always decreases the sugar of a normal serum, ca 10%.

(2) The influence of cations on the dynamic equilibrium of the serum sugar,  $\epsilon$ , after the addition of a physiological quantity (200 mg per 100 cc) acts exactly in the same way, but more intensely, NaCl does not influence the loss of the added dextrose, KCl decreases the loss considerably,  $\text{CaCl}_2$  and  $\text{MgCl}_2$  also decrease the loss, but less than KCl,  $\text{FeCl}_3$  always increases the loss of dextrose.

(3) Influence of anions on the static equilibrium of the serum sugar. The neutral sodium salts of the anions have been added in the same amounts as used in the cations (n/10 solutions, 0.02 cc to 1 cc of serum). Chloride and carbonate do not influence the serum sugar, phosphate and sulphate liberate sugar in a similar way as KCl,  $\text{Na}_2\text{SO}_4$  has a stronger action than  $\text{Na}_3\text{PO}_4$ .

(4) Influence of anions on the dynamic equilibrium of the serum sugar. The only salt which had a marked influence was  $\text{Na}_2\text{SO}_4$ , which decreased the loss of dextrose considerably.

(5) Influence of cations on the static equilibrium of the urea of the serum.  $\text{FeCl}_3$  has a pronounced decreasing influence upon the urea of the serum,  $\text{CaCl}_2$  has no influence,  $\text{MgCl}_2$  and also KCl do not influence, as a rule, the original urea of the serum, there are, however, considerable differences among the different sera.

(6) Influence of cations on the dynamic equilibrium of urea

tion of the first appearance of vasodepressing substance indicates that an appreciable time is required to develop vasodepressing substances in the peritoneal content. The control washings from normal dogs and from those in which peritonitis was not present had no effect upon the blood pressure of another dog.

(b) *Bile Peritonitis* The centrifuged exudate or the combined exudate and washings removed from 8 dogs in which bile peritonitis was present had no depressing effect on the blood pressure of other dogs while in a single instance vasodepression was noted with a fluid obtained from an animal that had died a few hours prior to lavage of the peritoneal cavity.

(c) *Extracts of Peritoneal Exudates, Washings and Sediments* Extracts of these materials prepared according to the method of Chang and Gaddum<sup>9</sup> demonstrated the presence and concentration of a vasodepressing substance in the whole exudate and in the centrifuged sediments at all times, both from the bile peritonitis animals and those with suppurative peritonitis. A vasodepressant extract was obtained from the supernatant centrifuged fluid only when that fluid itself contained such substances. Bacterial sediments contained no vasodepressing substance. The finding of such a substance in normal mammalian tissues is in agreement with the finding of such substances by Harkins and Harmon.<sup>9</sup>

(d) *Bacterial Filtrates* Without exception a vasodepressing substance with a delayed time of action of 20 to 45 minutes after injection was present in Mandler filtrates of *Escherichia coli*.

7535 P

### Mutual Influence of Crystalloids of Human Blood Serum on Their Equilibrium

ERNEST A. PRIBRAM

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The blood serum is an aqueous (ultramicroscopic)-suspension of colloids in a solution of crystalloids. The crystalloids are under normal conditions in a well balanced equilibrium, which is sustained by the colloids' static equilibrium. A normal blood serum for

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<sup>9</sup> Harkins, H. N., and Harmon, P. H., *Proc. Soc. Exp. Biol. and Med.*, 1934, **32**, 6

## 7536 P

## Urea, Promoter of the Catalytic Action of Blood Serum on a Specific Dextrose-Phosphate Reaction

ERNEST A. PRIBRAM.

*From the Loyola University School of Medicine, Department of Bacteriology and Preventive Medicine, Chicago*

Dextrose and phosphates have a high tendency to react. They are both present in animal and human tissue as well as in the blood serum. When mixed in an aqueous solution in the same quantities, which are present in the blood serum, *i. e.*, 3 mg of P in the form of a neutral phosphate and ca 100 mg of dextrose per 100 cc., we find a very slight reaction, which can be assumed because we recover a little less of dextrose and a little less of phosphorus than we do when the chemicals are added separately, 28 mg of P are recovered instead of 3 mg per 100 cc., and 90 mg of sugar instead of 100 mg. The same quantities added to human serum of a healthy individual react much more intensely. Instead of the original quantities added we recover only 25-50% of dextrose and ca 70% of phosphorus. The loss of dextrose has been explained in the previous paper. It is caused by the increase of dextrose, which is counteracted by the serum colloids. The loss of phosphorus, however, does not occur if no sugar is added. In this case we recover 100% of the added phosphate. Urea added to the serum does not influence the serum phosphate, and also does not influence the dynamic equilibrium of the phosphate, when this salt (3 mg per 100 cc.) and at the same time urea (30 mg per 100 cc.) are added. We still recover the entire phosphate. The reaction between dextrose and phosphate, however, is intensely accelerated and intensified, if phosphate, urea and dextrose are added to the serum. In this case only a small quantity of the phosphate, 10%, and frequently none of the added salt can be recovered, provided the serum is that of a normal healthy individual. The loss of the dextrose is not influenced by the urea, but there is a loss of the urea as high as 30-50%, which, as stated previously is caused by the adding of dextrose.

Pathological conditions, in which the physiological reaction of dextrose with phosphate remains incomplete will be considered in another article.

The catalytic action of the serum colloids and their promotion by the urea are specific for dextrose. Other saccharides, such as galactose or lactose, do not react with the phosphate, but allow 100% of



Urea added to the serum can be quantitatively recovered, there is no loss like in dextrose.  $\text{FeCl}_3$  causes a loss of the urea added, the other cations do not influence the dynamic equilibrium.

(7) Influence of anions on the static equilibrium of urea.  $\text{Na}_2\text{CO}_3$  causes a loss of ca. 10%,  $\text{Na}_2\text{SO}_4$  and  $\text{Na}_3\text{PO}_4$  cause a loss up to 50% of the original urea of the serum.

(8) Influence of the anions on the dynamic equilibrium of the urea. The 3 sodium salts examined all had the same influence on the urea, causing the loss of about 7% of the urea added.

(9) There is an interesting antagonism between the serum dextrose and the serum urea, concerning their influences on cations and anions. Adding of dextrose increases the influence of cations and nullifies the influence of anions on urea. Adding urea decreases the influence of cations on dextrose, nullifies the influence of  $\text{Na}_2\text{SO}_4$ , but intensifies the influence of  $\text{Na}_2\text{CO}_3$  and of  $\text{Na}_3\text{PO}_4$  on dextrose.

(10) Influence of non-electrolytes on non-electrolytes (mutual influence of dextrose and urea). Adding dextrose to the serum causes a loss of dextrose, as mentioned, as high as 75%. It does not, however, influence the serum urea (static equilibrium). There is no loss of urea, when urea is added (30 mg per 100 cc), and none of dextrose. The simultaneous addition of urea and dextrose does not alter the loss of dextrose which remains 75%, but causes a loss of the added urea (dynamic equilibrium) between 30 and 50% in a normal human serum.

All these experiments have been checked in aqueous solutions. The presence of the serum colloid is essential for the results. According to previous studies of the author<sup>1</sup> it is very likely that the struggle for water between the highly hydrophilic and even hygroscopic electrolytes and non-electrolytes plays an important rôle in the maintenance of their equilibrium in the presence of serum. The quantities added were exceedingly small: 0.15 mg of KCl, 0.32 mg of  $\text{FeCl}_3$  have been added, but even higher dilutions—0.03 mg of  $\text{FeCl}_3$  proved to be effective. The importance of these studies for the "Micrometabolism" (Wright<sup>2</sup>) in different diseases will be reported elsewhere.<sup>3, 5</sup> Anabolic and catabolic influences of drugs can easily be determined with these methods, *in vitro* as well as *in vivo*.

<sup>1</sup> Pribram, E., *Z. f. Kolloidchemie, Beih.*, 1911, **2**, 1.

<sup>2</sup> Wright, F., *Clin. Med. and Surg.*, 1933, **40**, 517.

<sup>3</sup> Pribram, *Arch. f. Gewerbepathologie*, 1934, **5**, 23.

Pribram, *Schweizer Wochenschr.*, 1934, in print.

cholesterol was followed by the same method as in the first group of animals. The results are summarized in Table II

TABLE II  
Blood Cholesterol in Sensitized Animals

Observed	Female 3400 gm.	Female 3150 gm.	Male 3250 gm	Female 3550 gm.
5 15	174	273	170	207
5 16	155	269	197	173
5 17	149	275	181	—
5-17	0.1 cc. 1/10 O T Intraven.	0.5 cc 1/10 O T Subcut.	1.0 cc. 1/10 O T Intraven.	1.0 cc 1/10 O T Subcut
5 18	255	330	375	156
5 19	325	415	675	415
5 21	373	547	266	357
5 22	200	—	145	110
5 23	231	347	162	150
5 24	195	168	110	100
5-25	148	165	107	83
5 29	215	225	150	165
6 1	250	235	153	230
6 3	275	287	265	150

The results indicate that these animals respond to the single injections of tuberculin with prompt though transient hypercholesterolemia. This is followed within a week by a return to normal value, which in some instances is first preceded by a fall below the normal level.

## 7538 C

### Bactericidal Power of Blood in Chronic Arthritis

FLOYD BOYS, F. D. GUNN AND S. J. LANG

From the Department of Pathology, Northwestern University Medical School and  
Evanston Hospital Arthritis Clinic

There is considerable evidence that chronic rheumatic joint disease, as well as acute rheumatic arthritis, is neither strictly a metabolic disturbance nor purely allergic in character but the result of hematogenous streptococcic infection of the joints. This evidence is of 4 types: the demonstration of streptococci in involved joints with typical structural alteration in direct relation to the actual distribution of the bacteria,<sup>1 2 3</sup> the streptococcemia which occurs at

<sup>1</sup> Forkner, C. E., Shands, H. R., and Poston, Mary A., *Arch. Int. Med.*, 1928, 42, 675.

<sup>2</sup> Cecil, R. L., Nicholls, E. E., and Stainsby, W. S., *Arch. Int. Med.*, 1929, 43, 571.

<sup>3</sup> Wetherby, M., and Clawson, B. J., *Am. J. Path.*, 1932, 8, 283.

the added phosphorus to be recovered, even when urea is added besides the sugar. It is evident that we deal here with an important anabolic influence of the urea, which, in the serum or tissues, is not merely a waste product, as it is as a rule considered in the urine.

7537

### Behavior of Blood Cholesterol Following Injections of Tuberculin

KATSUJI KATO (Introduced by B. Hamilton)

*From the Department of Pediatrics, University of Chicago*

The literature gives but scant attention to the relation of cholesterol to tuberculosis. It has been found by a few observers that the blood cholesterol is definitely decreased during the activity of the tuberculous process and this hypocholesterolemia, therefore, may be regarded as an index of poor prognosis. With the improvement of the pathologic process, however, the blood cholesterol rises and during convalescence it may be increased above the normal level. The present experiments endeavor to study the behavior of total blood cholesterol in rabbits following injections of old tuberculin.

A total of 10 rabbits were divided into 2 groups. The first group of 6 rabbits (average weight 1660 gm.) received single injections of 1:10 dilution of old tuberculin, and the blood cholesterol was determined daily by the Bloor method as modified by Sackett. The results are summarized in Table I.

The second group of 4 rabbits (average weight 3340 gm.) were first sensitized by repeated injections of old tuberculin over periods varying from 10-60 days. After rest periods of 2-6 months, these animals were given single injections of tuberculin and the blood

TABLE I  
Blood Cholesterol in Unsensitized Animals

Observed	Female 1725 gm.	Female 1400 gm.	Male 1750 gm.	Female 1725 gm.	Female 1725 gm.	Female 1650 gm.
5 26	138	113	100	118	120	150
5 28	202	212	225	—	275	200
5 29	192	300	187	202	170	267
5 29	0.2 cc 1/10 O T		1 0 cc. 1/10 O T		3 0 cc 1/10 O T	
	Intraven	Subcut.	Intraven	Subcut	Subcut.	Intraven
5 30	313	380	300	332	375	288
5 31	201	282	283	289	203	220
6 1	190	267	287	240	197	250
6-2	195	325 (1)	195	193	203	215
6 3	193	260	267	214	195	312

An eleven-year-old strain of *Streptococcus viridans* obtained from Dr B J Clawson was used as the test organism chiefly because it had been used previously in our routine agglutination tests. Several of the strains that we have isolated from the blood of arthritic patients have been similar culturally and serologically to this one. Tests with recently isolated strains of streptococcus gave unreliable results by our method.

For the test 1 cc of a suspension containing about 10,000 bacteria per cubic centimeter was used. This was added to 3 cc of a 50% dilution of serum, or to 3 cc of defibrinated blood, in a 7 cc Wassermann tube. The tube was closed with a paraffined rubber stopper and placed in the rack of a mixing machine in a 37° incubator. Constant agitation was found to be necessary for these tests and a rotating machine was devised for this purpose.\*

The degree of reduction of living organisms was determined by the plating method. For this a 0.5 cc sample of the mixture was removed, plated with 10 cc of dextrose agar immediately after the mixture was made and again after 4 hours and 8 hours of incubation in the agitating machine. The colony counts were made after 18 to 24 hours of incubation and recorded numerically when possible, or estimated roughly as 2 plus ( $4000 \pm$ ), 3 plus ( $6000 \pm$ ) and 4 plus ( $8000 \pm$ ) when too numerous for actual counting.

The results of the initial tests in arthritic and non-arthritic patients are summarized in Table I. For the sake of simplicity we have merely indicated as having bactericidal power those cases which showed inhibition after 4 hours of rotatory agitation in the incubator. The results varied from complete inhibition to slight (10%) reduction in the number of colonies.

In 38 arthritic patients the serum alone of only 2 cases (5%) exhibited bactericidal power, while in 3 cases (9%) among the controls definite bactericidal action was observed.

It is interesting that in the group of cases of active arthritis, all of which were of a severe type, only one blood failed to show definite inhibition of the strain used. The agglutination titer in these cases was well within normal limits and the sedimentation rates showed about the same average values and varied within narrower limits than in the normal cases.

In the group of 12 cases of arthritis that showed unequivocal clinical evidence of improvement, only 5 gave positive bactericidal tests. The sedimentation rates varied within the same limits as in the normals. Agglutination titers were not done at this time.

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\* Details of the technique and a description of the agitating machine are included in an accompanying paper.

intervals during the active phases of the disease,<sup>3, 4, 5</sup> the presence of streptococcic antibodies in arthritic patients in concentrations greater than those usually found in normal individuals,<sup>5, 6, 7, 8</sup> and the production experimentally of lesions in the joints of animals by the use of intravenous injections of streptococcus cultures.<sup>2, 10</sup> On the other hand, other investigators have failed to corroborate some of these observations.<sup>11-13</sup>

This positive evidence has led to the wide use of agglutination and sedimentation tests as diagnostic procedures in chronic arthritis and to the use of vaccines for treatment of the disease. Our own experience and that of others<sup>7</sup> with the use of agglutination tests and determinations of sedimentation rates have led us to believe that these have little practical value in diagnosis or as indices of the results of treatment. In searching for a more reliable index of immunity to streptococcic infection, we have tested the whole or defibrinated blood of 30 patients and the serum of 38 patients from the Evanston Hospital Arthritis Clinic for bactericidal power. As controls, 31 tests on the defibrinated blood and 22 on the serum of normal individuals were done. Bactericidal tests were performed also on the blood and serum of 10 patients suffering from non-streptococcic infections and in patients with acute streptococcic sepsis. In most instances the sedimentation rate and the agglutinating titer were determined on samples of blood drawn at the same time for comparison with the results obtained in the bactericidal tests.

Preliminary experiments showed that there was little difference between the bactericidal power of heparinized blood and that of defibrinated blood if the defibrination was done carefully and with constant technique. The latter method was finally adopted for routine tests because the procedure was simple and inexpensive.

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<sup>4</sup> Richards, J. H., *J. Am. Med. Assn.*, 1925, **84**, 637

<sup>5</sup> Gray, J. W., Fendrick, E., and Gowen, C. H., *Texas State Med. Journal*, 1932, **28**, 317

<sup>6</sup> Burbank, R., and Hadjopoulos, L. G., *J. Am. Med. Assn.*, 1925, **84**, 637

<sup>7</sup> Dawson, M. H., Olmstead, Miriam, and Boots, R. H., *J. Immunol.*, 1932, **23**, 187

<sup>8</sup> Clawson, B. J., Wetherby, M., Hilbert, E. H., and Hilleboe, H. E., *Am. J. Med. Sc.*, 1932, **184**, 758

<sup>9</sup> Keefer, C. S., Meyers, W. K., and Oppel, T. W., *J. Clin. Invest.*, 1933, **12**, 267

<sup>10</sup> Burbank, R., *Bull. N. Y. Acad. Med.*, 1929, **5**, 176

<sup>11</sup> Nye, R. N., and Waxelbaum, E. A., *J. Exp. Med.*, 1930, **52**, 885

<sup>12</sup> Dawson, M. H., Olmstead, Miriam, and Boots, R. H., *Arch. Int. Med.*, 1932, **119**, 173

<sup>13</sup> Bernhardt, H., and Hench, P. S., *J. Infect. Dis.*, 1931, **40**, 489

Of the 5 cases diagnosed as hypertrophic arthritis 3 gave positive bactericidal tests and the sedimentation rates were not significant

The acute and chronic non-streptococcic infections were local lesions, some with suppuration but none showing systemic effects. In 3 of the chronic cases the infection was localized. In these cases agglutination tests yielded normal values and the sedimentation rate, while slightly greater in the acute infections, varied within narrow limits. The bactericidal effect of the defibrinated blood in the more acute cases is comparable with that in the active cases of infectious arthritis. This evidence supports the idea that the bactericidal property of whole blood or defibrinated blood as applied to gram positive cocci is not specific.

Both of the cases of acute streptococcic sepsis exhibited marked bactericidal power. In both instances *Streptococcus viridans* was recovered repeatedly in blood cultures. It is interesting that in the patients with acute streptococcic or non-streptococcic infections and in the cases of active arthritis, the bactericidal power of the blood was approximately the same. The agglutination titers in these 2 patients with streptococcus sepsis varied within normal limits emphasizing again the lack of correlation between bactericidal power and the agglutination titer.

In the third division of the table the data accumulated from studies of 20 arthritic cases receiving intravenous vaccine therapy at the time of the initial bactericidal tests are summarized. As in past experience we find generally increased agglutination titers without correlation between titer values and clinical improvement. The 2 most active cases showed the lowest sedimentation rates. Vaccine therapy apparently had no effect upon either bactericidal power or sedimentation rate.

Conclusions from such small groups of cases as presented in the table must necessarily be guarded. However, we consider that the following generalizations are justified in the light of previous findings of other workers and supplementary unpublished data from our own experience.

- 1 Tests of bactericidal power of blood by the method used are of little or no value in the diagnosis of chronic arthritis.

- 2 In general, the defibrinated blood from active cases of atrophic arthritis and acute streptococcic and non-streptococcic infections more often exhibits inhibitory properties against *Streptococcus viridans* than the blood from normal individuals from patients with

TABLE I  
Bactericidal Power, Agglutination Titer and Sedimentation Rate in Arthritis and Non arthritic Conditions

	Total cases	Cases with pos. Bc tests	Agglutination Titer				Sedimentation Rate in mm (60 min)			
			Av in pos cases	Extremes in pos. cases	Av in neg cases	Extremes in neg cases	Av in pos cases	Extremes in pos cases	Av in neg cases	Extremes in neg cases
Normal individuals	31	21 (68%)	96	20 320	44	20 180	11	2 27	15	5 24
Arthritis (untreated)										
Atrophic { Recovering	12	5 (41%)					17	9 26	14	6 24
and mixed { Active	12	11 (91%)	50	20 80			11	2 23	20	5 21
Hypertrophic	5	3 (60%)					17	14 19	11	5 21
Arthritis (treated vaccine)										
Atrophic { Recovering	12	7 (58%)	1730	320 5120	1664	160 5120	18	4 29	12	6 10
and mixed { Active	2	2 (100%)	1600	640 2560			4	3 5		
Hypertrophic	6	3 (50%)	320	160 640	3630	640 5120	16	14 19	12	5 21
Streptococic sepsis	2	2 (100%)	160	0 320						
Non strep infections										
{ Acute	6	5 (83%)	104	0 320		80	14	4 28		28
{ Chronic	4	3 (75%)	50	20 80			10	3 18		6

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- 1 Tests of bactericidal power of blood by the method used are of little or no value in the diagnosis of chronic arthritis.

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chronic non-streptococcic infections, from quiescent or recovering cases of atrophic arthritis, or from hypertrophic arthritis

3 Intravenous streptococcic vaccine therapy does not influence the bactericidal property of blood while the agglutinating titer may be greatly increased

4 There is no correlation between streptococcic bactericidal property, agglutinating titer and sedimentation rate in the cases so far examined

5 Serum alone usually shows little or no bactericidal power against *Streptococcus viridans*, irrespective of its agglutinating titer. In exceptional cases there may be definite inhibitory action

## 7539 C

## Importance of Continuous Agitation in Bactericidal Tests with Streptococci

S. J. LANG AND FLOYD BOIS (Introduced by F. D. Gunn)

From the Department of Pathology Northwestern University Medical School and Evanston Hospital

Although the advantages of continuous agitation in phagocytosis experiments had been noted by earlier investigators,<sup>1 2 3</sup> Robertson and Sia<sup>4</sup> were apparently the first to use the rotatory-oscillation method extensively. All have observed that constant mixing during incubation promotes bactericidal action and produces more constant results than stationary incubation or intermittent agitation. Most of the previous work has been done with cultures of pneumococci and we were faced with the necessity of checking these factors with the streptococcus with which we were working.

An agitating machine utilizing the same principle as that in the machine used by Fenn<sup>2</sup> and later in the improved machine of Robertson and Sia<sup>4</sup> was designed and built for us by Mr. Wm. H. Hamilton, E. E. It consists of 3 brass flanges mounted rigidly on a motor driven shaft. Two of the flanges act as tube holders, having 5/8 in. perforations bored at uniform intervals near the periphery while the third flange acts as a guard. The flanges will hold 18 tubes at

<sup>1</sup> Rosenow, E. C., *J. Infect. Dis.*, 1906, **3**, 683

<sup>2</sup> Fenn, W. O., *J. Gen. Physiol.*, 1920, **3**, 439

<sup>3</sup> Kite, G. L., and Wherry, W. B., *J. Infect. Dis.*, 1916, **16**, 109

<sup>4</sup> Robertson, O. H., and Sia, R. H. P., *J. Exp. Med.*, 1924, **39**, 219

one time and the angle of the tubes can be altered by placing one end of the tube in the second or third perforation from that opposite the one containing the other end of the tube. The tubes are held in place by a rubber band which encircles them. A constant speed electric fan motor furnishes the power which is transmitted by pulley through a short belt (rubber band) and a worm and circular gear.

The rotating rack revolves at a constant speed of 18 r p m and the mixtures shift from the base of the tube to the top against a paraffined rubber stopper and back again during each revolution. The chief advantages of this design are the mechanical simplicity, constant speed and small space occupied by the machine. It fits conveniently on the top shelf of a small incubator and requires no more attention than occasional oiling of the motor (Fig 1)

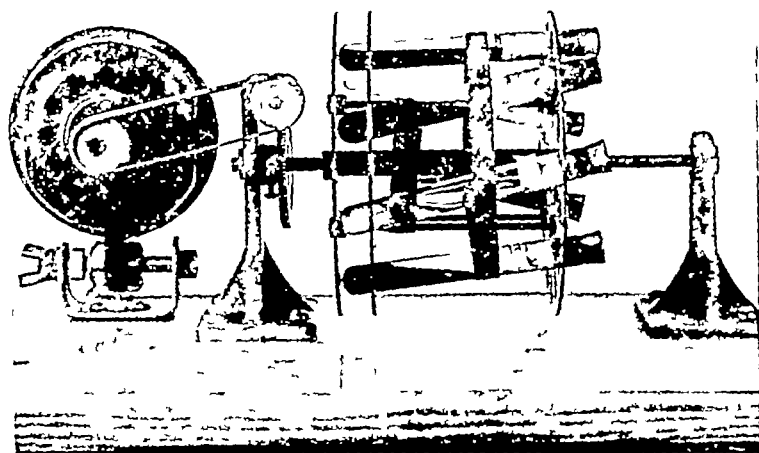


Fig 1

Tests were performed with defibrinated blood and washed suspensions of 24-hour cultures of *Streptococcus viridans*, with and without agitation in the machine. The mixtures were made in the proportion of 3 cc of freshly drawn, defibrinated blood and 1 cc of gelatin-Locke's solution containing about 10,000 bacteria. A 0.5 cc sample of this mixture was plated with 10 cc of dextrose agar immediately and again after 4 hours of incubation. The results are indicated in Table I. In the stationary tubes an actual reduction in the number of bacteria as indicated by the colony count was noted in only 2 cases of the 19 tested. In every case a much greater killing effect was obtained in the mixtures that were rotated in the machine.

TABLE I  
Bactericidal Effect With and Without Rotatory Agitation

Case No	Colony Increase or Decrease in %	
	Stationary	Rotatory Agitation
1 (F W)	+100	-100
2 (K S)	+1220	+59
3 (Z W)	+290	-78
4 (T S)	+1330	-98
5 (W F)	+1330	+1140
6 (A H)	+1000	+44
7 (D C)	+2000	+100
8 (L H)	+540	-99
9 (A F)	+2000	+690
10 (M S)	+2000	+260
11 (M K)	-75	-100
12 (W H)	+44	-75
13 (R H)	+322	-100
14 (M S)	+270	-100
15 (M B)	+110	-100
16 (G M)	+51	-100
17 (C C)	+127	+80
18 (A R)	-68	-100
19 (A A)	+200	+122

Similar differences were noted in 7 tests in which, instead of incubating in stationary tubes after the initial thorough mixing, the tubes were mixed by inverting several times by hand at 15-minute intervals and allowed to settle in racks in the interim (Table II)

TABLE II  
Bactericidal Effect with Intermittent Shaking and Rotatory Agitation.

Case No	Colony Increase or Decrease in %	
	Intermittent Shaking	Rotatory Agitation
1 (F L)	+60	-100
2 (D P)	+100	-100
3 (G E)	-12	-100
4 (E M)	+300	-99
5 (J E)	-56	-100
6 (Q L)	+2000	+43
7 (M S)	+1000	-99

In every instance the colony count was less in mixtures rotated constantly in the agitating machine

<u>Bactericidal</u>	
Case	
No	
1	(F W)
2	(K S)
3	(Z W)
4	(T S)
5	(W F)
6	(A H)
7	(D C)
8	(I H)
9	(A F)
10	(M S)
11	(M K)
12	(W H)
13	(R H)
14	(M S)
15	(M B)
16	(G M)
17	(C C)
18	(A R)
19	(A A)

Similar differences were observed when the tubes were mixed by inversion at regular intervals and allowed to settle.

<u>Bactericidal Effect with Intermittent Mixing</u>	
Case	
No	
1	(F.L.)
2	(D P)
3	(G.E.)
4	(E M.)
5	(J E)
6	(Q L)
7	(M S)

In every instance the colony count was determined in the agitating machine.

An intestinal extract was prepared by water extraction of the duodenum and first few inches of the ileum of a dog. The extent of digestion was practically identical with that caused by combined action of commercial trypsin and erepsin. The addition of these enzymes produced no further cleavage. No digestion of the carbohydrate portion of the molecule was observed.

These studies indicate that mucin, in contrast to most proteins, is relatively resistant to enzymatic hydrolysis *in vitro*, a property in accord with its ascribed protective action. However, it seems probable that further digestion of mucin occurs in the digestive tract. This is indicated by the fact that normally no readily detectable quantities of mucin are excreted from the intestinal tract, and, furthermore, that glucuronic acid of mucin is available for conjugation. Perhaps specific enzymes exist for this purpose, or some definite but at present unknown sequence of enzymatic action is required. These studies are being continued with purified enzyme preparations.

## 7541 C

### Depressor Extracts of Some Human Tissues.\*

HENRY N. HARKINS AND PAUL H. HARMON (Introduced by Edmund Andrews)

*From the Department of Surgery, The University of Chicago*

The recent work of Dale Dudley and their associates<sup>1 2 3</sup> has thrown new light on the depressor substances that may be extracted from animal tissues. The methods of preparation of and differentiation between the various depressor substances have been described especially by Chang and Gaddum<sup>1</sup>. Because these methods are rather new they have not yet been applied extensively to human tissues. The present work was undertaken to determine if there were any unusual amount of depressor substance in (1) carcinomatous tissue and in (2) toxic thyroid tissue.

All specimens were obtained from living patients during a sterile surgical operation. The specimens were extracted in 3 hours or less

\* Miller, C. O., Brazda, F. G., and Elliott, E. C., *Proc. Soc. Exp. Biol. and Med.* 1933, **30**, 633. Miller, C. O., and Connor, J. A., *Ibid.* 1933, **30**, 630.

\* Work done in part under a grant from the Douglas Smith Foundation.

<sup>1</sup> Chang, H. C., and Gaddum, J. H., *J. Physiol.*, 1933, **70**, 255.

<sup>2</sup> Dudley, H. W., *J. Physiol.*, 1933, **70**, 249.

<sup>3</sup> Euler, U. S., and Gaddum, J. H., *J. Physiol.*, 1931, **72**, 74.

absence of these conditions, identical material can be obtained by alcohol precipitation, either with or without additional peptic digestion. The mucin used in these investigations was prepared from an undiluted commercial preparation by precipitation in 70% alcohol. Mucin prepared in this manner was entirely resistant to further peptic action. It is quite possible that commercial mucin represents merely the peptic resistant portion of native mucin, though no conclusive evidence is available on this point.

In studying the action of trypsin upon mucin, enzyme preparations from 3 different commercial sources were employed. One sample was tested and contained, in addition to trypsin, the ereptic enzymes dipeptidase and aminopolypeptidase. The data obtained with all 3 preparations were practically identical, the amounts of amino nitrogen liberated being equal to 7.57%, 7.90%, and 7.85% of the total nitrogen of the mucin.

The commercial erepsin employed was found to contain, in addition to erepsin, a small amount of proteinase. When added to a tryptic digestate of mucin, the additional amino nitrogen liberated was equal to approximately 3% of the total nitrogen, or a total increase of approximately 11% due to the combined action of trypsin and erepsin.

Table I summarizes the results when cleavage was followed by means of 70% alcohol precipitation and subsequent analysis of the precipitated material. These data, in agreement with those obtained by the Van Slyke method, indicate partial digestion by both trypsin and erepsin.

TABLE I  
%Precipitation, Nitrogen and Reduction of Mucin Digestates

	% Precip *	% N	% Reduction† (as glucose)
Before digestion	83.2	7.15	34.5
After tryptic digestion	76.5	6.9	38.2
Tryptic + ereptic digestion	73.4	6.2	44.2

\* By alcohol at 70% concentration

† After acid hydrolysis

Proteolytic enzymes were also prepared from yeast by water extraction and their action upon mucin tested. An increase in amino nitrogen equal to 15.1% of the total nitrogen was observed.

The following enzymes were tested with negative results: maltase (alpha glucosidase), emulsin (beta glucosidase), steapsin and pancreatic amylase.

An intestinal extract was prepared by water extraction of the duodenum and first few inches of the ileum of a dog. The extent of digestion was practically identical with that caused by combined action of commercial trypsin and erepsin. The addition of these enzymes produced no further cleavage. No digestion of the carbohydrate portion of the molecule was observed.

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<sup>2</sup> Dudley, H. W., *J. Physiol.*, 1933, **70**, 249.

<sup>3</sup> Euler, U. S., and Gaddum, J. H., *J. Physiol.*, 1931, **72**, 74.

following operation and were kept in a refrigerator at 4°C in the meantime. The tissue was weighed and placed in 10% trichloroacetic acid (2 cc of acid for each gm of tissue). It was then cut up in the acid with scissors and left with occasional stirring for several hours. The extract was filtered through paper on a Buchner funnel and the tissue washed with 7% trichloroacetic acid. The filtrate was then shaken 3 or 4 times with ether in a separating funnel until it was only faintly acid to congo red paper. It was then concentrated at low pressure at 37°C until 1 cc of extract corresponded to about 1 gm of tissue. This solution was made neutral to congo red solution by titrating with 1-10 N NaOH and then used in the biological test.

In the present work the extracts were tested for effect on dog's and rabbit's blood pressure. The blood pressure was measured by a mercury manometer connected to a cannula in the right carotid artery. The extract was injected by means of a burette connected with a cannula in the left femoral vein of the dogs or the left external jugular vein of the rabbits. The depressor action was compared with that produced by a standard solution of acetylcholine, made up so that 1 cc corresponded to 1 gamma acetylcholine. If 1 cc of the unknown extract gave a fall in blood pressure equal to that produced by 1 cc of the standard solution then the unknown extract was said to contain 1 gamma acetylcholine equivalent per cc. An effort was then made to determine whether or not the depressor action was due to acetylcholine or some other substance. According to Chang and Gaddum acetylcholine is quite inactive when tested on the blood pressure of an atropinized rabbit. As will be shown later in the present work most of the tissues extracted produced quantitatively as great a fall in blood pressure of a rabbit after atropinization as before. Control injections of pure acetylcholine in these rabbits produced no fall in blood pressure after atropinization. This indicates that in the extracts tested using the atropinized rabbit's blood pressure as a criterion, acetylcholine was not present in large amounts.

According to Chang and Gaddum histamine produces little fall in rabbit's blood pressure. In the present studies the tissue extracts were just as active on the blood pressure of rabbits as on that of dogs, both when compared as to absolute dose per unit body weight and as to acetylcholine-equivalent. This indicates that in the extracts tested, using this criterion of Chang and Gaddum histamine was not present in large amounts.

Euler and Gaddum<sup>2</sup> found large amounts of a substance they



called the "P" substance in certain animal extracts. This substance lowers the arterial blood pressure of the atropinized rabbit. It can be differentiated from adenosine which is stable in alkalis and inhibits the rabbit's intestine. Wilson, Stewart and Harkins,<sup>4</sup> working in Wilkie's laboratory found that the depressor substance in the skin of burned and normal rabbits answered the specifications of the P substance and that acetylcholine was not present in anything but small amounts.

**Results** Six human tissues were extracted as follows: (1) Carcinoma of thyroid, woman aged 33 years, basal metabolic rate minus 6 before operation. (2) Quadriceps muscle from amputated leg. (3) Hemorrhagic fluid from aseptic necrosis of large nodule in adenomatous goitre, man aged 60 years, basal metabolic rate plus 21 five days before operation and plus 13 three days before operation. (4) A supposedly normal thyroid gland removed for cardiac disease, man aged 43, basal metabolic rate plus 7 before operation. (5) Pectoralis major muscle from radical mastectomy. (6) Toxic thyroid gland, woman aged 27 years, basal metabolic rate plus 24 just before operation, plus 43 a week previous.

The acetylcholine-equivalents of these extracts are given in Table I. Of the 5 extracts tested before and after atropinization 2

TABLE I

The acetylcholine equivalent of six human tissue extracts. The use of the term acetylcholine equivalent does not imply that the substance tested is actually acetylcholine.

Extract	Before atropine			After atropine		
	No. of animals used in assay		Aver ac equiv $\gamma$ per gm	No. of animals used in assay		Aver ac equiv $\gamma$ per gm
	Dogs	Rabbits		Dogs	Rabbits	
1 Carcinoma of thyroid	3	3	1.2	2	2	1.1
2 Quadriceps muscle	2	2	2.0	1	1	1.4
3 Thyroid cyst fluid	1	2	0.02	0	0	—
4 Normal thyroid	1	0	8.0	1	0	0.0
5 Pectoralis muscle	2	1	1.8	1	0	0.3
6 Toxic thyroid	2	2	3.6	1	1	5.6

showed only a slight change, 2 were less active and one more active after atropine. For the 2 that were less active after atropine, this conclusion was based on only a single assay in each case. It must be remembered, however, that after atropine, the blood pressure often falls somewhat and the absolute fall produced by an equivalent amount of substance may be less even though the relative fall is as great. The average acetylcholine-equivalent of the 2 normal human

<sup>4</sup> Wilson, W. C., Stewart, C. P., and Harkins, H. N., Depressor Substances in Burned Tissues, to be published.

following operation and were kept in a refrigerator at 4°C in the meantime. The tissue was weighed and placed in 10% trichloroacetic acid (2 cc of acid for each gm of tissue). It was then cut up in the acid with scissors and left with occasional stirring for several hours. The extract was filtered through paper on a Buchner funnel and the tissue washed with 7% trichloroacetic acid. The filtrate was then shaken 3 or 4 times with ether in a separating funnel until it was only faintly acid to congo red paper. It was then concentrated at low pressure at 37°C until 1 cc of extract corresponded to about 1 gm of tissue. This solution was made neutral to congo red solution by titrating with 1-10 N NaOH and then used in the biological test.

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Euler and Gaddum<sup>3</sup> found large amounts of a substance they

## 7542 C

## Rôle of Leucocytes and Serum in Streptococidal Activity of Blood

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In testing the defibrinated blood and serum of patients and normal individuals for killing effect against streptococci we found that, while the majority of samples of blood exhibited bactericidal power, the serum alone rarely caused reduction in the number of bacteria. This finding supported the idea that the effective bactericidal agent in whole blood acting against streptococci resides in the cellular elements and confirms the earlier observations of several workers, notably Mackie, Finklestein and Van Rooyden<sup>1</sup> with regard to gram positive cocci in general. Most of the previous work on gram positive bacteria, however, has been done with pneumococci<sup>2</sup> and staphylococci<sup>3</sup> and we considered it worth while to check and extend the work with streptococci.

Leucocyte counts were made at the time of withdrawal of samples of blood and repeated after defibrination was completed. The defibrination was accomplished by rotating the blood with glass beads in an Erlenmeyer flask under sterile precautions. In 6 samples of freshly drawn blood, leucocyte counts of 6930 to 9550 per cu. mm. were obtained and in the same samples after defibrination the leucocyte number was between 5200 and 7160, an average reduction

TABLE I  
Effect of Aging and Chilling on Bactericidal Test

Case No	Colony Count Bc Test			Leucocytes
	Start	4 hrs	8 hrs	
		%		%
1 Fresh blood	256	6 (-97)	320	6150
Chilled "	200	960 (+380)	8000±	4850 (-21)
2 Fresh blood	256	31 (-88)	2080	5300
Chilled "	245	8000± (+3200)	8000±	2435 (-54)
3 Fresh blood	448	8000± (+1700)	8000±	5940
Chilled "	320	8000± (+2400)	8000±	2825 (-52)
4 Fresh blood	140	5 (-97)	15	6400
Chilled "	110	800 (+630)	8000±	2600 (-59)
5 Fresh blood	175	0 (-100)	50	6600
Chilled "	160	3 (-98)	576	5350 (-19)

<sup>1</sup> Mackie, J, Finklestein, M H, and Van Rooyden, C E, *J Hyg*, 1932, **32**, 494

<sup>2</sup> Robertson, O H, and Sia, R H P, *J Exp Med*, 1924, **39**, 219

<sup>3</sup> Thalheimer, W, and Colwell, C, *J Lab and Clin Med*, 1929, **24**, 441

muscle extracts was 1.9, that of the toxic thyroid was 3.6, the normal thyroid was 8.0, the carcinoma of the thyroid was 1.2, and the fluid from the thyroid cyst was 0.02. These results indicate that in these single instances, there is no unusual amount of depressor substance in toxic thyroid tissue or in carcinomatous tissue.

*Controls* In addition to comparing the effects on atropinized and unatropinized dogs and rabbits with those produced by standard acetylcholine solutions, controls were made as follows: (a) 10% trichloroacetic acid alone was found to produce a definite depressor action roughly 0.5% ac-equiv per cc acid. (b) 10% trichloroacetic acid was extracted in exactly the same way as the human tissues. This included all steps of the process. Part of this solution was extracted with ether twice, part 4 times and part 6 times. All of these solutions were equally inactive, roughly less than 0.02% ac-equiv per cc acid. (c) Several of the tissue extracts were assayed without accurate neutralization after extraction and compared with the effects after neutralization to Congo red solution and to phenolphthalein. In general these extracts were equally active. (d) Extract No. 5 (pectoralis major muscle) was assayed before and after being passed through a Mandler filter and found to be equally active in both instances.

There are many substances isolated from animal tissues that fall under the classification of depressor substances. These include (a) histamine, (b) acetylcholine, (c) choline and other choline esters, (d) adenosine, (e) P substance, (f) potassium, and (g) R substance<sup>1</sup>. The biological assay used in the present paper indicates that the extracts of human tissues tested contained practically no histamine and very little acetylcholine because they lowered the blood pressure of atropinized rabbits. The various other substances were not definitely excluded, but nothing was found to indicate that the active principle was other than the P substance. The amount of depressor substance in any of the human tissues tested was not as great as that found in certain animal tissues such as the spleen of the horse or ox which Chang and Gaddum found to contain 4 to 30% ac-equiv. This amount was said to be actually due to acetylcholine itself. These authors also found 28% ac-equiv in extracts of a human placenta.

*Conclusions* In several human tissue extracts, including toxic thyroid tissue and carcinomatous tissue, depressor substances were found not to be present in unusual amounts. The major part of the depressor substance present in these tissue extracts does not act like acetylcholine or histamine when tested for effect on the blood pressure of atropinized rabbits.

factor This was found to be the case in each of 5 samples from different patients where the blood was fortified by the addition of leucocytes at the end of 4 hours of incubation The volume of leucocyte suspension added to each tube was equal approximately to that at the beginning of the test (Table III)

TABLE III  
Bactericidal Power after Fortification with Leucocytes

Case No	Control			Fortified at 4 hours		
	Start	4 hrs	8 hrs	Start	4 hrs	8 hrs
1	520	8000±	8000±	560	8000±	1216
2	80	160	4000±	90	175	115
3	2880	8000±	8000±	2900	8000±	1940
4	240	19	160	250	28	15
5	152	4	200	160	7	30

In a similar group of cases the blood samples were each divided into 2 equal portions One was run as usual as the control and the other was subjected to rotatory agitation in the incubator for 4 hours before the suspension of bacteria was added The killing effect of the blood was completely destroyed in 3 samples and seriously impaired in the other 2 as a result of the preliminary incubation (Table IV)

TABLE IV  
Reduction of Bactericidal Power after Preliminary Incubation

Case No	Control			4 hrs Preliminary Incubation		
	Start	4 hrs	8 hrs	Start	4 hrs	8 hrs
1	288	0	58	300	728	8000±
2	432	0	0	400	368	124
3	800	13	62	780	8000±	8000±
4	820	125	548	800	8000±	8000±
5	592	11	0	610	8000±	8000±

The mode of destruction of the bacteria and the rate at which they were removed were determined by the examination of stained films made from the mixtures at intervals during the tests For this experiment suspensions containing about 200 million bacteria per cc were used Blood films were made in the usual manner from a drop removed by sterile capillary pipette and stained with Wright's blood stain The relative numbers of polymorphonuclear cells containing bacteria and those showing no bacteria in their cytoplasm at the different intervals are recorded in Table V Toward the end of the 4-hour period the leucocytes were so few that only 10 or 15 leucocytes were found by systematic examination of the entire film In each case the majority of the leucocytes contained phagocytosed

of 23% This reduction was not sufficient to produce an appreciable alteration of the bactericidal power of the blood as proved by previous experiments After rotatory agitation for periods of 4 hours without the addition of bacteria, however, the reduction in number of intact leucocytes was from 70% to 90%

The effect of standing and refrigeration is illustrated in Table I, which gives the comparative colony counts from bactericidal tests made on fresh samples and samples allowed to stand in the refrigerator overnight These were incubated with the test culture for periods of 4 and 8 hours in the agitating machine In each blood sample that exhibited definite killing action when fresh, there was marked reduction of bactericidal power after refrigeration In these samples the degree of reduction in number of leucocytes varied between 19% and 59% It is obvious that the numerical reduction in leucocytes is not the most important factor The phagocytic activity of the intact leucocytes must have been seriously impaired by such treatment

Next a small series of tests was made to determine whether or not some of the inhibitory effect of the blood would remain after complete removal of the leucocytes Four samples of defibrinated blood from different patients were divided into 2 portions and one part centrifuged at low speed to throw down the formed elements The buffy coat was removed as completely as possible with sterile pipettes and parallel tests were run on these samples and on the complete defibrinated blood as controls The results (Table II) indicate

TABLE II  
Effect of Removal of Leucocytes

Case No	Colony Count (Control)			Colony Count (Leucocytes removed)		
	Start	4 hrs	8 hrs	Start	4 hrs	8 hrs
1	2880	8000±	8000±	2180	8000±	8000±
2	2240	37	86	2200	8000±	8000±
3	190	0	0	210	8000±	8000±
4	200	1	160	215	8000±	8000±

that after the more or less complete removal of leucocytes blood no longer inhibits the growth of streptococci In case 1, however the blood failed to show any inhibitory effect even in the control sample

The apparent exhaustion of bactericidal power after about 4 hours of incubation and the demonstration of marked reduction of leucocytes in mixtures that had been incubated for a few hours with rotatory agitation led us to suspect that the loss of killing power was due merely to exhaustion and disintegration of the leucocytes If true, an addition of fresh leucocytes should restore the exhausted

same sample (Table VI) No correlation was observed One of the two samples of blood showing the least bactericidal power, contained the highest concentration of complement and the 2 samples having the greatest killing power yielded very low complement titer

Finally the thermostability of the active factor was tested by removing the serum as completely as possible from samples of defibrinated blood after centrifugation and heating it at  $55^{\circ}\text{C}$  for 30 minutes The inactivated serum was then restored to the cells from which it had been removed and bactericidal tests run For controls we used a part of the same sample, subjected to the same treatment with the exception that the serum was not heated In a few cases a part of the serum was heated to  $68^{\circ}$ , returned to the cells and then tested for killing effect Similar tests were made with serum alone after heating at  $55^{\circ}$  or  $68^{\circ}$  for 30 minutes and with unheated controls In none of these cases was there any evidence that the bactericidal property was impaired by the heating of the serum

*Conclusions* The power of defibrinated blood to inhibit the growth of or destroy streptococci is dependent upon the presence of surviving leucocytes and is roughly proportional to the number of leucocytes, other factors being equal A minimal number of leucocytes is necessary for the demonstration of the bactericidal effect The bacteria are removed by phagocytosis and finally destroyed by intracellular lysis Complement is not necessary for the bactericidal action of defibrinated blood or serum The bactericidal element in defibrinated blood which is effective against streptococci is not destroyed by heating the separated serum at  $55^{\circ}\text{C}$  for 30 minutes The results of a small number of tests suggest that the heating of the serum even at  $68^{\circ}\text{C}$  does not impair the bactericidal effect of the restored defibrinated blood

## 7543 P

### Development of Gastric Ulcers and Decrease in Reducing Power of Adrenals Following Injection of Bile Salts.

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Sellards<sup>1</sup> reported the development of acute gastric ulcers in guinea pigs following intraperitoneal bile salt injection His ob-

TABLE V  
Rate of Phagocytosis in Bactericidal Tests

Case No		Start	½	1	1½	2	3	4	8 hrs
1	Pmn with bac	3	6	12	13	25	15	10	
	No intrac bac	22	19	13	2	0	0	0	
	Col in Bc test	100						20	64
2	Pmn with bac	5	8	18	25	25	18	15	
	No intrac bac	20	17	7	0	0	0	0	
	Col in Bc test	80						160	6000±
3	Pmn with bac	10	23	25	25	25	20	10	
	No intrac bac	15	2	0	0	0	0	0	
	Col in Bc test	150						1	1
4	Pmn with bac	4	11	15	24	25	15	15	
	No intrac bac	21	14	10	1	0	0	0	
	Col in Bc test	190						160	640
5	Pmn with bac	12	40	44	46	50	50	50	
	No intrac bac	38	10	6	4	0	0	0	
	Col in Bc test	200						1	1

cocci by the end of one or one and one-half hour and in 2 hours all of the cells contained bacteria in various stages of disintegration. No significant differences in rate of phagocytosis were observed in those blood samples exhibiting marked inhibitory power in the bactericidal tests and those showing less power.

In preliminary experiments it was found that while the presence of serum was necessary for the obtaining of bactericidal effects, dilutions up to 1-25 were apparently as effective as undiluted serum. The agglutinating titer had been found to bear no constant relationship to the bactericidal power of whole blood, defibrinated blood or serum. The effect of varying complement concentration remained to be determined. In 10 cases the complement titer and bactericidal power of the defibrinated blood were determined on the

TABLE VI  
Bactericidal Power and Complement Titer

Case No	Serum Dilution	Beginning Lysis cc	Complete Lysis cc	Colony Count in Bc Test		
				Start	4 hrs	8 hrs
1	1:50	2	0.5	160	2	368
2	1:50	1	0.2	340	1420	8000±
3	1:50	2	1.0	250	31	2080
4	1:25	5	0.8	240	0	0
5	1:25		0.3	288	23	350
6	1:50	5	1.0	240	8000±	8000±
7	1:50	5	1.0	240	3	450
8	1:50	9	1.0	152	4	200
9	1:25	7	0.5	250	10	50
10	1:50		1.0	192	0	0



C, should reveal any marked decrease in this substance. Unfortunately at the time these experiments were conducted, we did not have available 2,6-dichlorophenolindophenol, which, according to recent work, exhibits considerable specificity for ascorbic acid.<sup>7</sup> The technique consisted in grinding the whole adrenal under 10% trichloroacetic acid, filtering, and washing the residue, followed by titration of the filtrate with 0.002 N iodine, using starch as the indicator. For titration the left adrenal was generally employed, the right having been used for silver nitrate staining. From Table I it

TABLE I.  
Iodine Titration of Adrenals Following Bile Salt Injection.

Sex	Wt. gm.	Dose bile salts	Time for death	Wt adrenal gm	cc I <sub>2</sub>	cc I <sub>2</sub> /gm adrenal
M	234	control	killed	065	65	10.0
M	226	"	"	065	61	9.4
F	187	"	"	034	35	10.3
F	251	"	"	074	75	10.1
M	265	"	"	042	62	14.8
M	291	"	"	047	50	10.6
F	254	"	"	053	77	14.5
M	188	05 g	4¼ hrs.	061	40	6.6
M	167	.10	5¼	073	61	8.3
M	207	.10	2¼	075	16	2.1
M	200	.15	1¼	059	50	8.5
F	187	.10	4¼	038	40	10.5
F	175	.15	1¼	050	27	5.4
F	205	.15	2½	067	20	3.0
F	—	.15	2½	076	48	6.3
M	312	.05	26	066	53	8.0
M	286	.10	4	064	63	9.8
M	304	.15	2	075	57	7.6
F	304	.10	4	052	38	7.3
F	292	.10	4½	082	69	8.4
F	290	.15	2	067	46	6.9
M	332	10 initial				
		05 23 hrs				
		05 27 "				
		10 29 "	30½	067	56	8.3
F	294	05 initial				
		05 23 hrs				
		05 26 "	31	102	80	7.8

Average cc. iodine/gm. adrenal controls = 11.4  
injected pigs = 7.2

will be observed that injected animals showed a decreased iodine titration.

A series of 26 rats was also injected with bile salts. Doses comparable with those used with guinea pigs usually caused death within 24 hours, but in no case was ulceration similar to that produced in guinea pigs observed. Occasionally slight, superficial

<sup>7</sup> Birch, T. W., Harris, L. J., and Ray, S. N., *Biochem J.*, 1933, 27, 590

servations have been confirmed and extended by Tashiro and co-workers<sup>2</sup> We injected approximately 40 pigs with varying amounts of bile salts (Fairchilds) The expected individual variations in susceptibility were observed, but injections of 0.1 gm or more generally caused death and extensive ulceration of the gastric mucosa The latter was usually so marked that the site of the ulcers could be plainly observed from the outer surface of the stomach, appearing as thin, semi-transparent areas In 3 animals perforation occurred, gastric contents being found in the peritoneal cavity at autopsy

Szent-Gyorgyi<sup>3</sup> reported the darkening of the adrenal cortex when subjected to a silver nitrate solution Harris and Ray<sup>4</sup> and Siehrs and Miller<sup>5</sup> report that this does not occur in guinea pigs on a scorbutic diet When we attempted to stain, with silver nitrate, the adrenals of pigs previously injected with bile salts, darkening was slight or absent Adrenals of apparently normal pigs may however, not stain with silver nitrate Gough and Zilva<sup>6</sup> report that in pigs given 10 cc of decitrated lemon juice daily for a period of 3 months the adrenals did not stain, although at autopsy no abnormalities were observed We kept 12 pigs on a diet of oats, an occasional carrot, and 2 cc of orange juice daily for one week The cortex of the adrenals did not stain with silver nitrate The absence of this staining reaction may merely indicate an insufficient excess of vitamin C in the diet to allow for its deposition in the adrenal Therefore, in the remaining experiments, a diet abundant in vitamin C was administered, the pigs receiving oats, fresh carrots and cabbage, and, in addition, 4 cc of orange juice daily for a period of 3 weeks prior to injection The adrenals of control animals stained almost totally black with 0.4% silver nitrate solution when exposed for 3 minutes to a 115 watt blue mazda lamp at a distance of approximately 8 inches The adrenals of animals injected with bile salts also showed some reduction under similar treatment, but in practically all cases the extent of darkening was distinctly less than in the controls and tended more toward a brown than to the black of the controls The decrease in reducing power was also confirmed by iodine titration, which, although not specific for vitamin

<sup>1</sup> Scallards, A. W., *Arch Int Med*, 1909, **4**, 502

<sup>2</sup> Tashiro, et al., *Med Bull Univ Cincinnati*, 1931, **6**, 110, 124, 130, 134, 144

<sup>3</sup> Szent-Gyorgyi, A., *Biochem J*, 1928, **22**, 1387

<sup>4</sup> Harris, L. J., and Ray, S. N., *Biochem J*, 1933, **27**, 303

<sup>5</sup> Siehrs, A. E., and Miller, C. O., *Proc Soc Exp Biol. and Med*, 1933, **30**,

<sup>6</sup> Gough, J., and Zilva, S. S., *Biochem J*, 1933, **27**, 1279

Other ciliates examined, *Frontonia*, *Spirostomum*, *Dileptus*, show differentials in staining and reduction essentially similar to those of *Paramecium*, but with different ranges of concentration and different degrees of decrease in oxygen. The animals and other material from the infusion decrease the oxygen sufficiently to permit more or less reduction in *Frontonia* and *Dileptus* in open preparations, while *Spirostomum* requires several hours in sealed preparation for complete reduction after slight staining.

In *Hydra* tentacles stain first with concentrations used thus far and if staining is not carried to the point of injury each tentacle shows a basipetal reduction gradient in low oxygen. With further staining before reduction loss of reducing power occurs first at the tentacle tip and progresses basipetally as staining proceeds and cytolysis follows basipetally. In the body ectoderm reduction occurs most rapidly in the hypostome region and progresses basipetally, provided staining has not progressed to the point of injury and loss of reducing power. The basal stalk region of *Pelmato-hydra* reduces more rapidly than the body after light staining, but is also more susceptible than the body and injury and loss of reducing power occur with comparatively little staining. In *Stenostomum* chains of zooids the head regions of the developing zooids beyond a certain stage of development reduce the dye more rapidly than more posterior levels with light staining, but are more susceptible to loss of reducing power after deeper staining. The ventral body wall also reduces more rapidly than the dorsal. In the microdrilous oligochetes, *Tubifer* and *Nais* the body wall of the anterior region and the posterior growing region consisting of a large number of developing segments do not stain or stain less rapidly than the middle region and the ventral body wall does not stain or stains less rapidly than the dorsal in low concentrations of dye in the oxygen content of the cultures of decaying vegetation, bacteria, protozoa, etc., in which the animals are maintained in the laboratory. After staining up to a certain point anterior and posterior regions reduce more rapidly than the middle in low oxygen and the ventral body wall reduces more rapidly than the dorsal. With high dye concentrations the anterior end stains more deeply than the middle and its reducing power is decreased or lost. The posterior region is so susceptible that it is usually killed before it becomes very deeply stained.

Concentrations used in these experiments range from 1/5000 to 1/300000 methylene blue. Since the leucobase stains and injures some forms (e.g., *Paramecium*) much more rapidly than the oxidized dye the range of concentrations for certain results is lower

erosions were seen, but nothing comparable with the deep, extensive ulcers which developed in the guinea pigs was observed. When the rats' adrenals were stained with silver nitrate, the darkening was immediate and marked. At present we are unable to say definitely whether this resistance of the rat to ulcer production through bile salt injection is due to their ability to synthesize vitamin C or to some other species difference.

## 7544 P

## Differential Reduction of Methylene Blue by Living Organisms.

O. M. CHILD

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With the oxidized dye and with a leucobase prepared by adding small amounts of HCl and  $\text{Na}_2\text{S}_2\text{O}_3$  to dye solutions axial differentials or gradients in rate of reduction of dye have been observed in various unicellular and multicellular organisms. *Paramecium* is able to reduce methylene blue in mixtures of culture fluid and dye exposed to air, provided the animals are numerous in proportion to volume of fluid or gather in aggregations and decrease oxygen locally, or provided other organisms which take up oxygen are present, but reduces more rapidly in sealed preparations with small amounts of fluid. With high oxygen content of solutions the anterior ectoplasm stains more rapidly than other parts. In low concentrations of dye with somewhat lower oxygen content permitting some reduction stain appears first in the deepest part of the posterior entoplasm, extends anteriorly in the entoplasm and the ectoplasm does not stain or stains more slowly, also from posterior to anterior, except for the extreme posterior tip which often stains less rapidly than adjoining regions. Apparently rate of staining under these conditions varies inversely as reducing power of different regions. In stained but uninjured animals reduction first becomes evident in the ectoplasm of the anterior end and progresses posteriorly, somewhat more rapidly along the peristome than on the aboral side and the extreme posterior tip of some individuals shows early reduction. In high concentrations the anterior ectoplasm stains more rapidly and more deeply than other regions at first and with sufficient staining it is injured and its reducing power is decreased or lost while more posterior regions are still able to reduce the dye in low oxygen.

Other ciliates examined, *Frontonia*, *Spirostomum*, *Dileptus*, show differentials in staining and reduction essentially similar to those of *Paramecium*, but with different ranges of concentration and different degrees of decrease in oxygen. The animals and other material from the infusion decrease the oxygen sufficiently to permit more or less reduction in *Frontonia* and *Dileptus* in open preparations, while *Spirostomum* requires several hours in sealed preparation for complete reduction after slight staining.

In *Hydra* tentacles stain first with concentrations used thus far and if staining is not carried to the point of injury each tentacle shows a basipetal reduction gradient in low oxygen. With further staining before reduction loss of reducing power occurs first at the tentacle tip and progresses basipetally as staining proceeds and cytolysis follows basipetally. In the body ectoderm reduction occurs most rapidly in the hypostome region and progresses basipetally, provided staining has not progressed to the point of injury and loss of reducing power. The basal stalk region of *Pelmato-hydra* reduces more rapidly than the body after light staining, but is also more susceptible than the body and injury and loss of reducing power occur with comparatively little staining. In *Stenostomum* chains of zooids the head regions of the developing zooids beyond a certain stage of development reduce the dye more rapidly than more posterior levels with light staining, but are more susceptible to loss of reducing power after deeper staining. The ventral body wall also reduces more rapidly than the dorsal. In the microdrilous oligochetes, *Tubifer* and *Nais* the body wall of the anterior region and the posterior growing region consisting of a large number of developing segments do not stain or stain less rapidly than the middle region and the ventral body wall does not stain or stains less rapidly than the dorsal in low concentrations of dye in the oxygen content of the cultures of decaying vegetation, bacteria, protozoa, etc., in which the animals are maintained in the laboratory. After staining up to a certain point anterior and posterior regions reduce more rapidly than the middle in low oxygen and the ventral body wall reduces more rapidly than the dorsal. With high dye concentrations the anterior end stains more deeply than the middle and its reducing power is decreased or lost. The posterior region is so susceptible that it is usually killed before it becomes very deeply stained.

Concentrations used in these experiments range from 1/5000 to 1/300000 methylene blue. Since the leucobase stains and injures some forms (e.g., *Paramecium*) much more rapidly than the oxidized dye the range of concentrations for certain results is lower

than with the latter. The dye is more toxic in light, even the light of the microscope condenser, than in darkness.

That the results described are due to reduction, not to diffusion outward of the dye is shown by the fact that after reduction the color returns within a few seconds with increase in oxygen content of the fluid. It may also be noted that in all cases the reduction gradient in animals not irreversibly injured is essentially identical with the gradient of susceptibility to a large number of chemical and physical agents in gradually lethal concentration or dosage.

The anteroposterior reduction gradient in *Paramecium* was noted in an earlier paper.<sup>1</sup> Recently Roskin and Semenov,<sup>2</sup> using a leucobase only, have concluded from the observed course of reduction that oxidation occurs most rapidly in the posterior region of *Paramecium*. Their results can be duplicated by following their procedure but this procedure results in deeper staining of the anterior ectoplasm and less rapid or no reduction there while more posterior regions are still able to reduce, consequently they have failed to observe the normal anteroposterior reduction gradient.

## 7545 C

### Effect of 1-2-4 Dinitrophenol on Cellular Respiration

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From the Lasker Foundation for Medical Research and the Department of Medicine, the University of Chicago

Numerous investigators (Plantefol,<sup>1</sup> Field, Martin and Field,<sup>2</sup> Ehrenfest and Ronzoni<sup>3</sup>) have reported that 1-2-4 dinitrophenol added *in vitro* to plants, yeast cells and frog tissues increases their respiration. This increase seems to be associated with an increased aerobic fermentation in the case of yeast cells (Cutting and Tain-

<sup>1</sup> Child, C. M., and Deviney, E., *J. Exp. Zool.*, 1926, **43**, 257

<sup>2</sup> Roskin, G., and Semenov, W., *Z. f. Zellforschung u. mikr. Anat.*, 1933, **19**, 150

\* Rockefeller Foundation Fellow

<sup>1</sup> Plantefol, L., *Ann. Physiol. Physicochim. Biol.*, 1933, **8**, 127

<sup>2</sup> Field, J., 2nd, Martin, A. W., and Field, S. M., *Proc. Soc. Exp. Biol. and Med.*, 1933, **31**, 56

<sup>3</sup> Ehrenfest, E., and Ronzoni, E., *Proc. Soc. Exp. Biol. and Med.*, 1933, **31**, 318

<sup>4</sup> Cutting, W. C., and Tainter, M. L., *J. Pharm. and Exp. Ther.*, 1933, **48**, 410

ter<sup>4</sup>) and an acceleration of anaerobic lactate production in the case of frog tissues (Ehrenfest and Ronzoni)

The experiments here reported were performed in an attempt to determine the mechanism of this rise in cellular respiration. The oxygen consumption was measured with the Warburg apparatus. The yeast (baker's) was suspended in 0.066 M phosphates, the frog tissues, in frog Ringer's, buffered with phosphates to pH 7.46, the goose red cells in 0.9% NaCl buffered with phosphates to pH 7.46. All these solutions contained 0.2% glucose. Gonococci were suspended in 0.9% NaCl and buffered with phosphates to pH 7.01 and pH 6.0.

Field, Martin and Field's contention that dinitrophenol is active only in its undissociated form is not supported by the findings of Ehrenfest and Ronzoni, nor by our own experiments. Dinitrophenol increased the respiration of yeast at pH 6.64 (where the concentration of the undissociated form was only 0.0158 mg per liter), and the respiration of frog tissues and goose red cells at pH 7.46 (where dinitrophenol is practically wholly dissociated) (Table I).

TABLE I  
Effect of 1-2-4 Dinitrophenol on Respiration of Tissues and Cells

Tissue	Concentration of Dinitrophenol mg./L.	O <sub>2</sub> Consumption		% Increase
		Before D.N.P. c.mm./hr	After D.N.P. c.mm./hr	
Yeast pH = 6.64 T = 25°	50	121.8	151.6	20
		136.5	156.3	13
		140.5	155.1	9
		124.6	157.2	21
		137.3	184.7	26
		114.0	174.5	35
Frog Kidney pH = 7.46 T = 30°	5	21.2	37.8	44
		4.6	7.2	36
		12.0	20.4	41
		22.6	34.2	34
Frog Liver pH = 7.46 T = 30°	5	15.6	21.4	27
		15.8	24.6	36
		24.6	34.0	28
		11.0	12.8	14
Goose Red Cells pH = 7.46 T = 37°	7	73.6	113.5	54
		76.3	112.0	47
		77.7	119.3	54
	13	75.6	140.0	85
		82.3	140.0	70

The increase in cellular respiration observed after the addition of dinitrophenol seems not to be due to a direct oxidizing action of this compound on the oxidizable substrates. Thus dinitrophenol did not oxidize lactate activated by  $\alpha$ -hydroxyoxidase of gonococci, an oxida-

tion readily catalyzed by reversible dyes (Barron and Hastings<sup>5</sup>) nor did it oxidize linseed oil, an oxidation catalyzed by hemin (Robinson<sup>6</sup>). Furthermore, when cellular respiration was inhibited by the addition of specific inhibitors of the oxidizing enzymes, KCN and CO, there was no increase of respiration after addition of dinitrophenol. Indeed some decrease of respiration was observed upon the addition of dinitrophenol to cyanide-treated goose red cells. These experiments were performed with frog kidney and liver, and goose red cells in the case of cyanide (0.002 M) and frog kidney and yeast in the case of CO (Table II). We may add that the formation of the potassium salt of metapurpuric acid by the action of KCN on aqueous solutions of dinitrophenol, occurs only in alkaline reaction and at 60°. At 38° and pH 7.46 (the pH of our experiments) such a reaction does not occur.

Dinitrophenol showed no accelerating influence on oxidations produced by gonococci. As oxidizable substrates, glucose, lactate, and pyruvate were used (Table III).

TABLE II.  
Effect of 1,2,4-Dinitrophenol on Cells and Tissues after Inhibition of Respiration by KCN and CO

Tissue	Inhibitor	O <sub>2</sub> Consumption	
		Before D.N.P.	After D.N.P.
Frog Kidney	KCN	c.mm./30 min.	c.mm./30 min.
		2.5	1.0
		2.5	3.1
		5.0	4.9
		4.6	4.1
Frog Liver	KCN	3.3	2.6
		2.9	1.7
		7.3	3.5
		6.1	3.7
		6.4	6.0
Goose Red Cells	KCN	4.0	0.7
		17.9	10.8
		19.7	9.6
		31.1	6.8
		20.2	5.2
Frog Kidney	CO O <sub>2</sub> (96.5/3.5)	1.1	0.4
		0.4	0.4
		1.4	1.1
		1.3	0.9
Yeast	CO O <sub>2</sub> (90/10)	64.0	66.0
		66.2	64.6
		52.2	55.0
		64.2	65.2

<sup>5</sup> Barron, E. S. G., and Hastings, A. B., *J. Biol. Chem.*, 1933, 100, 165.

<sup>6</sup> Robinson, M. E., *Biochem. J.*, 1924, 18, 255.



TABLE III  
Dinitrophenol and Oxidations Produced by Gonococci.

Substrate	O <sub>2</sub> Consumption in 30 Minutes	
	Without D N.P	With D N.P
Glucose	234.8	234.8
Lactate	48.2	44.0
Pyruvate	130.0	91.9

*Conclusion* Since dinitrophenol is unable to oxidize such a labile compound as lactate activated by  $\alpha$ -hydroxyoxidase, is without effect when the respiration of cells and tissues has been inhibited by cyanide or carbon monoxide, and has no action on the respiration of certain bacteria, where the complicated controlling mechanisms present in highly organized cells are absent, it is concluded that the increase in respiration produced by dinitrophenol is not due to direct oxidation of the oxidizable substrates. It is suggested that dinitrophenol acts by combining with some of the substances acting as agents for the control of the speed of cellular oxidations, thus increasing the activity of the oxidizing enzymes.

## 7546 P

### Effect of Histamine and Alcohol on Acid Secretion of Stomach of Postoperative Cases

PAUL H. HARMON AND EDMUND ANDREWS

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In order to stimulate the secretion of acid by the stomach of postoperative surgical cases, the action of histamine and alcohol was tested. In 10 cases a Rehfuß tube was inserted through the nose

TABLE I  
Histamine Tests

No	Control		15 min		30 min.		45 min.	
	Free	Total	Free	Total	Free	Total	Free	Total
1	0	11	12	36	82	102	73	95
2	0	—	30	48	68	85	55	75
3	0	—	27	51	90	112	80	105
4	0	—	27	51	90	112	80	105
5	30	55	32	54	72	89	65	87
6	0	—	0	—	0	—	0	—
7	0	—	0	—	—	—	60	81
8	0	—	20	38	89	104	119	129
9	0	—	0	—	0	—	0	—
10	0	—	112	134	124	142	135	155

before complete recovery from the anesthesia and 2 to 5 hours later, 0.5 or 1.0 mg histamine given hypodermically

The results of these tests indicate that in many cases at least the impaired acid secretion may be readily remedied by histamine. The flow in 6 of the 10 cases was profuse and contained a high acid content. The reaction did not seem to be dependent on the type of operation or anesthetic as some of the minor operations failed to secrete while major operative cases often yielded high acid contents. One of those which failed to secrete acid had very large amounts of bile in the stomach which might have masked it but the other 2 did not.

Five similar tests were made with alcohol. The usual technique of the alcohol test meal was carried out, 50 cc sauterne 15% alcohol being injected through the nasal tube.

TABLE II  
Alcohol Tests

No	Control		15 min		30 min.		45 min.	
	Free	Total	Free	Total	Free	Total	Free	Total
1	0	—	40	63	48	70	35	53
2	0	—	0	—	0	—	0	—
3	0	—	0	—	0	—	0	—
4	0	—	56	83	110	112	56	72
5	0	—	0	—	0	—	0	—

Thus while but 2 of the 5 responded at all, in these two the acid reached very high titres.

It is clear, therefore, that in some cases at least the stomach of the usual postoperative case, in which achlorhydria is the rule, is capable of stimulation to secrete hydrochloric acid. There was obvious clinical benefit from the procedure. None of the patients vomited and none were nauseated. All were promptly given large amounts of solid food and appeared to have no discomfort. The contrast with the usual surgical patient at this stage of his convalescence was very marked.

*Conclusion* Histamine and alcohol have their usual action of stimulating gastric secretion in the postoperative surgical patient. Their administration is clinically beneficial.

## 7547 P

## Studies on Acholic Cachexia. V Pathological Changes \*

ALEXANDER BRUNSCHWIG, ARTHUR D BISSELL AND EDMUND ANDREWS

*From the Department of Surgery, The University of Chicago*

Many observers have reported extensive pathological changes in the organs of animals suffering from cachexia cholipriva. These have in general consisted of 3 processes, (1) degenerative changes in the liver, pancreas, heart and skeletal muscles, (2) osteoporosis, and (3) hepatitis, due to ascending infection from the cannula. Whether the first 2 types of changes are due to lack of bile in the system or due to ascending infection of the liver has not been clearly understood.

Our experience with bile fistulas made by various methods has been that ascending cholangitis was nearly always present, in some cases being of a severe type. The bile canaliculi were surrounded by rosettes of infiltrating cells and degeneration of the lobules of the liver was marked. However, with fistula made by the Dragstedt cannula, this has not occurred. The liver showed no signs of infection. The bile ducts were clear, no bile thrombi were found and even when the fistula had persisted for 5 months there were absolutely no infiltrations of leucocytes about the bile ducts, and no fibrosis. In these cases there was a corresponding absence of other signs of liver damage. Central necrosis, ordinarily seen over large areas did not occur. The cells stained clearly and contained no vacuoles or fat droplets. Iron stains showed that a small amount of pigments was hemosiderin and not bile pigment. This was also seen in the spleen but was not of a high degree. Normally, the dog has considerable pigment deposits of this type in the spleen and liver. Similarly, in spite of the extreme emaciation of the animals the other viscera were normal. The myocardium showed no waxy degeneration nor did the skeletal muscle. The intestinal and gastric mucosa was normal. Konjetzky gastritis was not present as has been reported previously, there being no eosinophiles in the submucous layers. The adrenals were normal, except for the total absence of any fat droplets, or lipid.

Not only in the series here reported, but in an extensive former series of bile fistulas, has osteoporosis been conspicuous by its ab-

\* Work done in part under the Jessie Horton Koessler Fellowship of the Institute of Medicine

sence The literature on this subject is highly contradictory Since Pavlov first reported osteoporosis with biliary fistulas, Looser<sup>1</sup> has found it to occur also with pancreatic and intestinal fistulas Seidel<sup>2</sup> reports similar findings In some reports the bone absorption was by means of osteoclasts and in others no lacunar absorption was noted although porosis was present Modern views are distinctly against the humoral absorption of bone, the so-called halisteresis Klinke<sup>3</sup> believed the bone atrophy was simply an accompaniment of general cachexia, and it is true that in most cases reported clinically this was extreme as in the recent paper of Wangensteen.<sup>4</sup> At any rate in a considerable series of fistulae we have not observed osteoporosis, as estimated either by X-ray or by histological demonstration of lacunar absorption Klinke estimates that about two-thirds as much calcium is lost in the bile as in the urine This loss hardly seems excessive More important is the recent demonstration that calcium may be absorbed from the intestinal tract in the absence of bile The obvious explanation seems to be that an infected bile fistula produces a marked acidosis Our dogs, not having an acidosis, had no porosis

*Conclusion* 1 In uninfected bile fistulas no degenerative changes in the liver occur nor does osteoporosis

7548 P

### Studies on Acholic Cachexia VI Bile Acid Factor \*

ARTHUR D BISSELL AND EDMUND ANDREWS

*From the Department of Surgery, The University of Chicago*

The classic studies of Whipple and his co-workers in this field have elucidated clearly the general problems of bile acid metabolism Our work is in general confirmatory of these, but certain other factors enter into the situation when the cachexia becomes extreme In no case has any ingestion of bile been permitted in our experiments and the analysis of the bile in the later cachetic stages brings out new points

<sup>1</sup> Looser, *Verhandl. deutsche Path. Ges.*, 1907, **11**, 291

<sup>2</sup> Seidel, *Munch. Med. Wochschr.*, 1910, **57**, 2034

<sup>3</sup> Klinke, *Klin. Woch.*, 1928, **1**, 385

<sup>4</sup> Wangensteen, *J. Am. Med. Assn.*, 1929, **93**, 1199

\* Work done in part under the Jessie Horton Koessler Fellowship of the Institute of Medicine

As has been the experience of all previous workers, the total output of bile acids varies over a wide normal range. It varies not only with the individual dog, the diet, the fluid intake and the general condition of the animal, but other unknown factors evidently cause even wider fluctuations. Careful examination of our records often fails to give any explanation of fluctuations amounting to 200-300%. In general the amounts expressed in milligrams per kilo per day were considerably less than those given by Whipple. He estimates that a normal 10 kilo dog keeps about 7-8 gm of bile salts per kilo in circulation, and that a fistula dog puts out 80-130 mg per kilo per day. As can be seen from our tables our dogs put out but 20-40 mg per kilo per day in the earlier stages, and when the cachexia became extreme often excreted only a small fraction of that amount.

TABLE I

Postoperative	Average Daily Mg B.A. for Period			
	Dog No 958 Wt. 19.5	Dog No 959 Wt. 17.5	Dog No 940 Wt. 18.8	Dog No 960 Wt. 17.5
Days				
6	223	210	1308	375
10	327	210	1308	375
15	398	528	1308	375
19	398	432	554	991
23	184	342	554	135
27	612	212	665	135
31	604	450	914	258
36	609	466	1237	288
40	383	713	1375	853
44	418	339	1137	583
48	401	211	646	295
56	580	393	1208	441
64	460	422	1096	551
72	540	422	976	197
99	622	422	278	609
114	105	192	61	—
130	105	194	61	—
150	402	194	—	—
167	602	128	—	—

The relation of the condition of the dog to the amount of bile salts excreted is not a direct one. Two of the 4 animals here reported excreted even more freely during the terminal stages. In our experience the opposite has more often been true. It is clear, however, that, if a very high excretion may in some cases be maintained right up to death from inanition, it is logical to assume that lack of bile salts in the body is not a major causative factor in the cachexia.

Both in these and in previous experiments, it has become evident that diet is not the only factor in regulating the bile salt output in cachexia. If as Whipple says the basic endogenous bile salts from

tissue waste amount to 30-40 mg per kilo per day and the rest is dietary, this mechanism does not necessarily work in the later cachectic stages. While in some cases the increase of meat in the diet may markedly increase the amounts of bile salts excreted, in others (dogs No 940 and 959) the opposite occurs. It is well known that a meat diet hastens the exitus of bile fistula animals and in many cases such as this the added intoxication resulting from the meat seems to bring about a closing down of the excretory mechanism.

*Conclusion* Acholic cachexia is not due to lack of bile salts in the body

## 7549 P

## Studies on Acholic Cachexia. VII Effect of Viosterol \*

EDMUND ANDREWS AND ARTHUR D BISSELL.

*From the Department of Surgery, The University of Chicago*

The rapid loss of weight, anemia and other changes which may occur in biliary fistula have recently been attributed to lack of ability to absorb fat-soluble vitamin. Takasu<sup>1</sup> has postulated that lack of bile salts in the intestinal tract prevents absorption of ergosterol in the same manner that fat digestion is impaired or stopped. Murakamis<sup>2</sup> reports that the excretion of bile acids is quadrupled by a single cubic centimeter of irradiated ergosterol, given subcutaneously. Others have found that vitamin administration had a markedly beneficial effect on the anemia and also prevented the bone changes.

Our experiences do not substantiate any of these conclusions. As shown in a previous article, the anemia did not seem an important factor. It was not profound and occurred mostly as a terminal phenomenon. Also the bone changes have not occurred in animals who did not have an ascending hepatitis. In 2 animals in whom frequent blood counts were made there was no effect from the administration of  $\frac{1}{2}$  cc viosterol (250 D per cc). In these experiments the viosterol was given about the third month and the fall in the hemoglobin and red count was more rapid after its administration.

\* Work done in part under the Jessie Horton Koesler Fellowship of the Institute of Medicine

<sup>1</sup> Takasu, M., *Deutsche Z f Chir*, 1930, **221**, 240

<sup>2</sup> Murakamis, R., *J Biol Chem*, 1928, **9**, 321

than before. As to the bone changes, much emphasis has been put by some workers on the lack of rise in blood calcium after viosterol administration to bile fistula dogs. The well known tolerance of the dog to viosterol is here overlooked. Its calcium threshold is low and viosterol injection even in very large doses does not affect the blood calcium even of the normal dog, so it would hardly be expected to act any better in the presence of bile fistula.

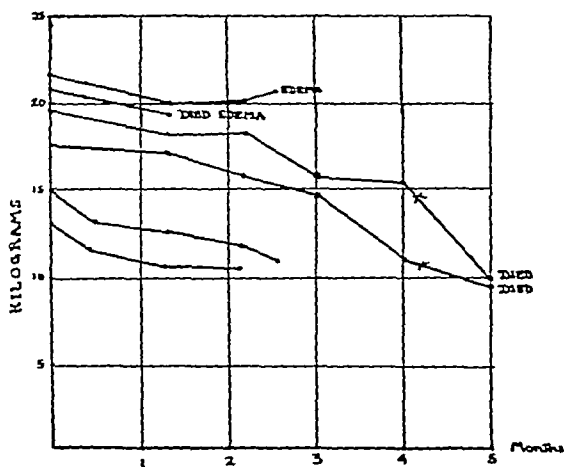


FIG 1

Effect of Viosterol in Bile Fistula Dogs

4 short experiments had viosterol 250 D 1 cc. every 4 days.

2 long experiments had viosterol 250 D  $\frac{1}{2}$  cc daily from point marked X.

Much of the previous work has been done on dogs which were allowed to lick their fistulae and such animals may be kept in good health for very long periods. It is rigidly guarded against as in our experiments, in quite an extensive series, the loss of weight has been irregular and at the rate of about 1-1.5 kilos a month. It is quite clear from the accompanying table that administration of viosterol even in very large doses has no effect whatsoever on the progress of the cachexia nor does it appear to save the life of any of the animals.

The completeness of the biliary fistula may be the cause of the apparent differences in our experiences from those of others. It is quite conceivable that an animal which gets minimal but adequate amounts of bile by mouth (5 cc. daily according to Whipple) is in a different condition from those having none. In the former condition viosterol may exert a profound effect which is absent in the latter.

*Conclusion* Viosterol does not exert a favorable influence on dogs with complete biliary fistulae

## 7550 P

Influence of Variations of O<sub>2</sub> and CO<sub>2</sub> Tension in Inspired Air  
Upon Hearing

ERNST GELLHORN AND IRWIN SPIESMAN

*From the Department of Physiology, College of Medicine, University of Illinois*

Although tests made on aviators and other observations at high altitudes indicate that O<sub>2</sub>-lack causes sensory disturbances,<sup>1</sup> particularly in vision, no systematic study seems to have been made in which the influence of various O<sub>2</sub>- and CO<sub>2</sub>-tensions was investigated in regard to sensory functions. Such a study seems to be of particular interest since the importance of these factors for various reflexes is well known. The present paper is based upon 96 audiometer experiments carried out on 6 thoroughly trained subjects. The administration of various O<sub>2</sub>-, N<sub>2</sub>- and CO<sub>2</sub>-air mixtures, which were inhaled from several large Douglas bags, was preceded and followed by control periods in which the threshold for a certain sound was determined in intervals varying between 1 and 4 minutes. Long control series extending over several hours were also carried out in order to determine the spontaneously occurring variations in threshold. The observation room was nearly sound proof.

In 37 experiments the influence of breathing CO<sub>2</sub>-air mixtures (2%-8.4%) during 5-22 minutes was studied upon the threshold of C 128, C 2048, and C 4096 cycles per second. It was found that a distinct hearing loss occurred during CO<sub>2</sub> breathing at and above 3% CO<sub>2</sub>. Depending on the CO<sub>2</sub> concentration and the duration of the breathing period, the recovery after the end of the CO<sub>2</sub>-period varies somewhat, but in general it was found that in less than 15 minutes the threshold was the same as before the experiment.

Contrary to expectation, similar losses in auditory acuity were obtained in 26 experiments in which the CO<sub>2</sub> tension of the blood was lowered by means of voluntary hyperpnea which was carried out for 3-6 minutes. The threshold determined immediately after the hyperpnea period during which apnea obtained was considerably

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<sup>1</sup> McFarland, R. A., *Arch Psychol*, 1932, 145, 1.



higher and a gradual return to normal was observed during the next 10-15 minutes

In a third group of 33 experiments 7.5%-15.8% O<sub>2</sub> was inhaled for 8-30 minutes. Here again it was found that during the period of O<sub>2</sub>-lack a decrease in hearing occurred. The recovery period, after readmission of air, depended greatly upon the degree of O<sub>2</sub>-lack produced during the experimental period. If 10% or less O<sub>2</sub> was inhaled for 15-30 minutes a decrease in hearing persisted for considerable periods of time, in some cases for several hours. During this time no other symptoms were present. The administration of high O<sub>2</sub> mixtures (50-60% O<sub>2</sub>) for 5-10 minutes does not seem to influence the course of the recovery period. These observations seem to indicate that a diminished O<sub>2</sub>-supply for relatively short periods of time produces changes in the nervous mechanism involved in hearing which are only slowly reversible. Furthermore, they indicate that O<sub>2</sub>-lack, CO<sub>2</sub>-excess, and CO<sub>2</sub>-lack influences hearing in the same fashion, although the effect of O<sub>2</sub>-lack is most severe. Another interesting phenomenon was observed in all 3 groups of experiments. The readmission of air leads in some cases to a considerable temporary improvement in hearing. Our observations agree with those of Schubert,<sup>2</sup> who found that after O<sub>2</sub>-lack upon readmission of air an improved visual discrimination is observed, as well as motor hyperexcitability. But in our experiments it is shown that such a temporary supernormal phase is not a specific reaction to O<sub>2</sub>-lack but also occurs after CO<sub>2</sub>-inhalation and voluntary hyperpnea.

#### 7551 P

### Influence of Variations of O<sub>2</sub> and CO<sub>2</sub> Tension in Inspired Air Upon After-Images.

ERNST GELLHORN, IRWIN SPIESMAN AND LESTER F. M. STORM.

*From the Department of Physiology, College of Medicine, University of Illinois.*

It was our objective to study the influence of variations of O<sub>2</sub> and CO<sub>2</sub> tension upon a simple and quantitatively measurable visual process in man. We chose the latent period of a negative after-image. The experimental subject fixated with both eyes the center of a yellow square on a grey background at a distance of 60 cm. The eyes were closed for one minute prior to each experiment.

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<sup>2</sup> Schubert, G., *Pflüger's Arch.*, 1933, **231**, 1.

*Conclusion* Viosterol does not exert a favorable influence on dogs with complete biliary fistulae

7550 P

### Influence of Variations of O<sub>2</sub> and CO<sub>2</sub> Tension in Inspired Air Upon Hearing

ERNST GELLHORN AND IRWIN SPIESMAN

*From the Department of Physiology, College of Medicine, University of Illinois*

Although tests made on aviators and other observations at high altitudes indicate that O<sub>2</sub>-lack causes sensory disturbances,<sup>1</sup> particularly in vision, no systematic study seems to have been made in which the influence of various O<sub>2</sub>- and CO<sub>2</sub>-tensions was investigated in regard to sensory functions. Such a study seems to be of particular interest since the importance of these factors for various reflexes is well known. The present paper is based upon 96 audiometer experiments carried out on 6 thoroughly trained subjects. The administration of various O<sub>2</sub>-, N<sub>2</sub>- and CO<sub>2</sub>-air mixtures, which were inhaled from several large Douglas bags, was preceded and followed by control periods in which the threshold for a certain sound was determined in intervals varying between 1 and 4 minutes. Long control series extending over several hours were also carried out in order to determine the spontaneously occurring variations in threshold. The observation room was nearly sound proof.

In 37 experiments the influence of breathing CO<sub>2</sub>-air mixtures (2%-8.4%) during 5-22 minutes was studied upon the threshold of C 128, C 2048, and C 4096 cycles per second. It was found that a distinct hearing loss occurred during CO<sub>2</sub> breathing at and above 3% CO<sub>2</sub>. Depending on the CO<sub>2</sub> concentration and the duration of the breathing period, the recovery after the end of the CO<sub>2</sub>-period varies somewhat, but in general it was found that in less than 15 minutes the threshold was the same as before the experiment.

Contrary to expectation, similar losses in auditory acuity were obtained in 26 experiments in which the CO<sub>2</sub> tension of the blood was lowered by means of voluntary hyperpnea which was carried out for 3-6 minutes. The threshold determined immediately after the hyperpnea period during which apnea obtained was considerably

<sup>1</sup> McFarland, R. A., *Arch. Psychol.*, 1932, 145, 1.

# Pacific Coast Section

University of California, June 21, 1934

7552 C

## Carbohydrate-Fibrinolytic Linkage in *Streptococcus hemolyticus*.\*

R. B. MADISON (Introduced by W. H. Manwaring)

From the Department of Bacteriology and Experimental Pathology, Stanford University, California.

The discovery by Lancefield<sup>1</sup> of a human-diagnostic "carbohydrate" fraction in certain pathogenic strains of *Streptococcus hemolyticus*, and Tillett and Garner's<sup>2</sup> demonstration of a specific fibrinolytic function in similar streptococci, suggests a possible genetic linkage between these 2 hereditary (or acquired) specific bacterial characters. To test this possibility, 189 strains of hemolytic streptococci previously titrated for their fibrinolytic function<sup>3</sup> have

TABLE I.

Lancefield Human-diagnostic Carbohydrate Titer of Fibrinolytic Streptococci

The cultures are grouped with reference to their quantitative fibrinolytic function. Specific carbohydrate titrations were made by the Lancefield technique (a) Ring test, 30 minutes, 37.5° C., (b) Dilution test, 18 hours, ice chest

Specific fibrinolytic titer		Human-diagnostic carbohydrate titer	
No. of strains tested	Lytic titer	Ring test (30 min.)	Dilution test (18 hr.)
A. <i>S. hemolyticus</i> of clinical origin			
1	++++	+++-	++++
17	-----	++-	++++
12	+++	++-	++++
15	++	++-	++++
2	-	++	----
100	0	0	+-
4	0	0	0
B. <i>S. hemolyticus</i> of veterinary origin			
3	+	0	=
38	0	0	0
C. <i>S. viridans</i> of clinical origin			
33	0	0	0

\* Work supported in part by CWA, in part by the Rockefeller Fund Research Fund of Stanford University School of Medicine

<sup>1</sup> Lancefield, R. C., *J. Exp. Med.*, 1933, **57**, 571

<sup>2</sup> Tillett, W. S., and Garner, R. L., *J. Exp. Med.*, 1933, **58**, 485

<sup>3</sup> Madison, R. B., *Proc. Soc. Exp. Biol. and Med.*, 1934, **31**, 1018

After a fixation time of 10 seconds the eyes were closed and a stop watch started. When the negative after-image appeared the watch was stopped. Control experiments showed that after a period of training the latent periods were very constant if the intervals between the individual experiments were at least 10 minutes. Fifty-five experiments were performed with 5 subjects. In the first group the influence of O<sub>2</sub> lack was studied by allowing the experimental subject to breathe various air-nitrogen mixtures from 7 to 30 minutes. The O<sub>2</sub> concentration varied between 9.2% and 16.0%. Whereas a reduction of the O<sub>2</sub> concentration to 13% was without influence on the latent period of negative after-images, very considerable changes occurred after breathing 9-11% O<sub>2</sub> for various times (7-27 minutes). The latent period was either considerably lengthened or became infinite since the after-image disappeared. In those experiments in which a negative after-image appeared, the subject noticed a decrease in its intensity. In some cases the latent period of the after-image remained lengthened even 10 minutes after the end of the breathing period. The subsequent experiments showed approximately the same latent periods as before the O<sub>2</sub>-lack experiment.

Similar experiments were carried out in order to study the influence of CO<sub>2</sub>. They showed that CO<sub>2</sub> is without effect upon the latent period of negative after-images when breathed in concentrations of 2-2.5% for as long as 26 minutes. The threshold concentration seems to be about 3-3.5%. A distinct increase in the latent period is observed in experiments with 4-7% CO<sub>2</sub> which was breathed for a period of 4-20 minutes. The effects were reversible.

A final series of experiments was carried out in order to study the influence of a reduction in the CO<sub>2</sub> tension upon sensory function. The subjects breathed with the rhythm of a metronome (between 64 and 90 per minute) for periods of 3-6 minutes. Immediately afterwards the after-images were investigated and showed regularly an increase in latent period.

The observations show conclusively that O<sub>2</sub> lack, CO<sub>2</sub> excess, and CO<sub>2</sub> lack have the same end effect on the sensory mechanism involved in the production of after-images. An interpretation of our experimental data in regard to the site of action will be postponed until more material on other sensory functions is available but it may be said that the effects are not due to circulatory disturbances. The blood pressure was either unchanged or varied only a few millimeters, whereas much greater alterations in blood pressure induced by physical exercise were without influence upon the latent period of after-images.

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been retitrated for their specific carbohydrate fraction by the Lancefield technic. Control tests were run with 2 human (C 203 and K 96) and 2 veterinary (P 454 and K 158 E) strains kindly furnished by Dr Lancefield. The results of these titrations are summarized in Table I.

Within the limits of the experimental error, there is an exact correlation between the Tillett-Garner specific fibrinolytic titer of *S. hemolyticus* and their Lancefield human-diagnostic carbohydrate titer by the ring test.

## 7553 C

### Immunological Types of Fibrinolytic Streptococci \*

J. K. VAN DEVENTER (Introduced by W. H. Manwaring)

*From the Laboratory of Bacteriology and Experimental Pathology, Stanford University, California.*

In order to test the possibility of there being more than one immunological type of fibrinolytic streptococci, 40 local strains of *Streptococcus hemolyticus* were titrated against various specimens of normal and immune human plasma-clot. To make these titrations parallel Tillett-Garner tests<sup>1</sup> were run with 1:1, 1:2, 1:4, 1:8, and 1:16 dilutions of 24-hour broth filtrates of the strains in question. The maximum dilution giving distinct fibrinolysis by the end of 24 hours was recorded as approximate lytic titer for a given blood sample. A preliminary series of duplicate tests showed that the experimental error in such titrations is not greater than one dilution either way from the recorded titer.

Data from 2 typical titrations are recorded in Table I. The 2 immune plasmas here recorded were drawn from convalescent cases, one of 90 days', the other of 12 months' duration.

Adopting the plasma-clot Van as the arbitrary standard, the table shows a normal range of fibrinolytic susceptibility of human blood varying from 4 times to 0.6 of the arbitrary standard.

There is apparently but one fibrinolytic type among the 40 streptococcus strains tested. The immune plasmas are consistently re-

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TABLE I.

Fibrinolytic titration of *S. hemolyticus*.

The table records the highest dilution of the 24-hour Chamberland filtrate giving distinct fibrinolysis

Strain No	Fibrinolytic titer with normal plasma-clots			Immune plasma clots	
	Rach.	Rel.	Van.	Con.	Sim.
135	16	8	2	0	0
10	8	4	4	1	0
28	8	4	4	0	0
95	8	4	1	0	0
93	4	2	1	0	0
46	4	4	0	0	0
168	1	0	0	0	0
175	1	0	0	0	0
41	0	0	0	0	0
Average titer	5.5	3	1.3	0.1	0
Relative susceptibility	4	2.3	(1)	0.1	0

Strain No	Fibrinolytic titer with normal plasma clots			Immune plasma clot Sim.	
	Muel.	Van.	Mad.	Till.	Sim.
30	16*	16*	16*	16	0
3	16*	16*	16	4	0
91	8	2	1	1	0
22	8	2	1	0	0
4	4	4	1	1	0
61	4	2	1	1	0
94	1	0	0	0	0
15	0	0	0	0	0
Average titer	7	5	4.5	3	0
Relative susceptibility	1.4	(1)	0.9	0.6	0

\*Dilutions above 1:16 not tested.

sistant to all strains. Within the limits of the experimental error (one dilution, plus or minus) all normal plasmas are consistently susceptible.

## 7554 P

### Intranuclear Inclusions in Brain of Chick Embryo after Inoculation of Egg with Virus of Equine Encephalomyelitis.

W. P. COVELL (Introduced by K. F. Meyer)

From the George Williams Hooper Foundation, University of California, San Francisco, Cal.

Recently intranuclear inclusions have been described by Hurst<sup>1</sup> in the nerve cells of animals suffering from equine encephalomyelitis.

<sup>1</sup> Hurst, E. W., *J. Exp. Med.*, 1934, 59, 529

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Within the limits of the experimental error, there is an exact correlation between the Tillett-Garner specific fibrinolytic titer of *S. hemolyticus* and their Lancefield human-diagnostic carbohydrate titer by the ring test.

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time than did the California strain. Inclusion bodies in the embryonic nerve cells were more numerous in the various regions of the brain with the former virus. No definite regions of the brain contained greater numbers of inclusions except larger nerve cells were more disposed to their formation. No intranuclear inclusion bodies were found in the embryonic nerve cells of the spinal cord. They made their first appearance after the ninth hour and were abundant in 18 to 24 hours. It is of interest in this regard to note that Higbie and Howitt found the virus to be present in the brain of the chick embryo in 9 hours and thereafter.

The inclusions are rounded acidophilic masses usually located nearer the periphery of the nucleus. They are variable in size, ranging from tiny bodies surrounded by an halo and occupying only a small part of the nucleus, to those which appear to make up one-fourth to one-third of the nuclear volume. Usually there is one inclusion body to a nucleus but more than one is by no means rare, they seldom exceed 2 in a single nucleus. There are in addition to inclusion bodies certain necrotic nerve cells which stain deeply with phloxine and bear a resemblance to nerve cells found in acute poliomyelitis. The nucleus of such a cell is shrunken, the marginated chromatin no longer stains blue and intranuclear inclusions may be present.

To determine if similar bodies appeared after inoculation of certain other neurotropic viruses into the 10-day incubated egg Borna disease and poliomyelitis viruses were utilized. Embryos and membranes were taken from a series of eggs every 6 hours over a period of 12-72 hours following inoculation of the eggs with the virus. In no instance were intranuclear inclusion bodies of the type found in the nerve cells of embryos infected with equine encephalomyelitis apparent in the brains of the embryos infected with Borna disease or poliomyelitis. The inclusions described by Hurst are similar in appearance to those in the embryonic nerve cells of the developing chick. The most obvious difference between the two is the relatively larger size of the inclusions in the embryonic nerve cells.

These bear a close resemblance to those occurring in Borna disease and in poliomyelitis. It becomes of interest to report the finding of inclusions in the brain of the chick embryo after inoculation of the developing egg with the virus of equine encephalomyelitis.

The procedure of inoculation of the developing chicks and membranes has been adequately described by Higbie and Howitt<sup>2</sup> as well as a study of the propagation and neutralization of the virus *in vivo*. Material obtained from this experimental work was fixed either in Zenker's fluid or in saturated corrosive sublimate containing 5% glacial acetic acid. The 2 to 5 micra sections of the membranes and embryos were stained with Giemsa or phloxine-methylene blue. Two series of sectioned embryos and membranes representing 3-hour intervals over a period of 24 hours were available for study. The New Jersey strain of virus and the California strain being represented in each of one series.

A noticeable edema of the chorio-allantoic membrane appeared in 3 to 6 hours following inoculation of the 10 to 12-day incubated egg. Normal horse serum was found to provoke a similar reaction but to less degree while physiological saline failed to produce an edema of the membranes. The swelling and gelatinous appearance of the membranes onto which the virus had previously been dropped rapidly increased and by 18 to 21 hours there was a collapse of many of the vessels and slight patchy streaking of the membranes. No definitely circumscribed areas of infection such as occur when herpes simplex is cultivated on the chorio-allantoic membrane (Dawson<sup>3</sup>) are seen.

Microscopically the membranes first show a thickening of the mesothelial layer and this subsequently becomes more prominent. There is no increase in the numbers of cells in this layer but the intercellular spaces are greater. In 9 hours there is a tendency towards ectodermal proliferation and slight inflammatory reaction. This process is more or less generalized, different areas revealing these reactions in varying degrees. In later stages a necrosis of the ectodermal layer with breaking up of the nuclear chromatin into irregular shaped blocks, ballooning of nuclei and necrosis of cytoplasm results. No intranuclear inclusion bodies are to be found in the cells of the membranes.

The brain of the embryonic chick is markedly softened, edematous and congested with prolonged cultivation of the virus. The New Jersey strain of the virus caused these changes in a shorter period of

<sup>2</sup> Higbie, E., and Howitt, B. F., in manuscript.

<sup>3</sup> Dawson, J. R., *Am. J. Path.*, 1933, 9, 1.

the number of cells or the quantity of protoplasm of the control tubes

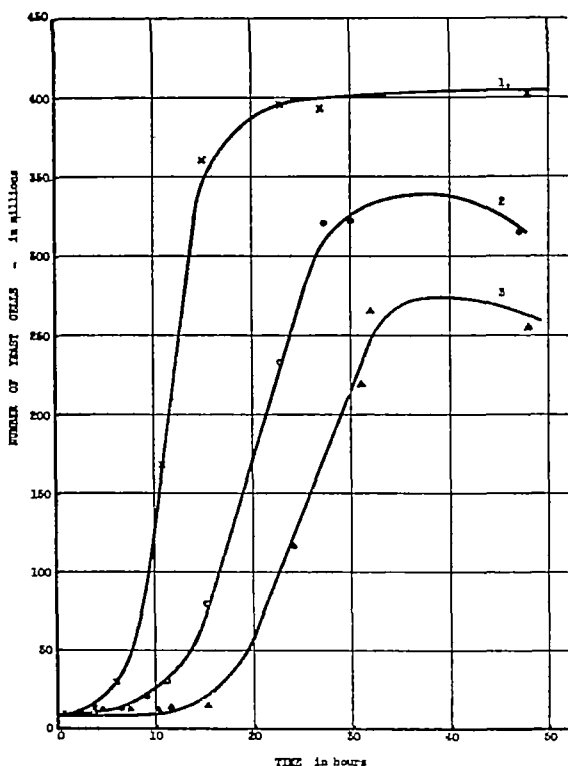


FIG 1

Comparison of lag and log phases of yeast growth from identical seedings. All tubes contain 5% glucose and 10% yeast autolysate made up in 0.2 M phosphate buffer, pH 6.8. Concentration of free acid alpha DNP is 0 in tube 1,  $1.795 \times 10^{-6}$  molar in 2,  $2.87 \times 10^{-6}$  molar in 3.

Testing the yeast grown in this manner in the Warburg respirometers we found that the alpha-DNP optimum for respiration has been shifted in each case to a lower value. This can probably be explained by residual alpha-DNP in the cells in spite of the repeated washings.

A striking effect, perhaps due to acclimatization or selection of yeast cells, or both (compare Fulmer<sup>6</sup>) is the greatly increased respiratory rate per  $10^8$  cells observed with yeast grown in tubes containing alpha-DNP. This may reach 300% of the control level. Further work is in progress.

<sup>6</sup> Fulmer, E. I., *J. Physical Chem.*, 1921, **25**, 455.

## Inhibition of Yeast Growth by 2-4 Dinitrophenol \*

A. W. MARTIN AND J. FIELD II.

*From the Department of Physiology, Stanford University*

The undissociated acid form of 2-4 dinitrophenol (alpha-DNP) has been shown by Field, Martin and Field<sup>1, 2</sup> to stimulate both the rate of oxygen consumption and of fermentation by yeast

Is this heightened metabolic level reflected in an increase in the growth rate? To test this we have grown yeast in the L-shaped rocker tubes designed by Fraser,<sup>3</sup> rocking 42 times per minute through an angle of 50°, in a water bath with temperature maintained at 25° C. The medium used was 10% yeast autolysate<sup>4</sup> in 0.2 M phosphate buffer at pH 6.8. The solution contained 2% to 5% glucose. Adequate precautions were taken to prevent contamination during inoculation and sampling, and our pure strain<sup>1, 2</sup> of *Saccharomyces cerevisiae*, race F, was used. Under these conditions there was little or no change in pH over a period of 4 days. Use of such yeast, tubes and media gave good checks (within 5%) in the control tubes.

The concentration of the sodium salt of alpha-DNP which gave maximum stimulation of respiration at pH 6.8 was 400 mg per liter or  $1.785 \times 10^{-3}$  molar<sup>1, 2</sup>. This gives a concentration of  $2.87 \times 10^{-3}$  molar for the free acid form. Instead of a stimulation of growth, a marked inhibition occurred with this dosage, there was an increase in duration of the lag and log phase (nomenclature of Buchanan<sup>5</sup>) and a decrease in the total population attained, as shown in Fig. 1. All of the free acid concentrations tried by us, ranging from  $7.18 \times 10^{-6}$  to  $7.18 \times 10^{-4}$  molar, had a more or less inhibitory effect on growth.

Hopkins tube readings agreed with the cell counts in showing that at no period did the tubes containing alpha-DNP attain either

\* Supported in part by a grant from the Rockefeller Fluid Research Fund of the Stanford University School of Medicine.

<sup>1</sup> Field, J. II., Martin, A. W., and Field, S. M., *Proc. Soc. Exp. Biol. and Med.*, 1933, **31**, 56.

<sup>2</sup> Field, J. II., Martin, A. W., and Field, S. M., *J. Cell and Comp. Physiol.*, 1934, **4**, 405.

<sup>3</sup> Fraser, C. G., *J. Physical Chem.*, 1921, **25**, 1.

<sup>4</sup> Orla Jensen, S., "The Lactic Acid Bacteria," Copenhagen, 1919.

<sup>5</sup> Buchanan, R. E., *J. Infect. Dis.*, 1918, **23**, 109.

anatomical lesions were slight but the histologic examination revealed very extensive and pronounced degenerations with inflammatory alterations in the central and peripheral nervous system

(2) A virus obtained from a fatal infection in Utah and the Argentine virus infect by the intracerebral route only. Filtrates injected intravenously or suspensions administered intracutaneously fail to incite the disease

(3) Thirty horses injected subcutaneously with 5 cc of a 20% suspension of California guinea pig passage virus had slight febrile reactions. Subsequently they tolerated large doses of Western virus

(4) Of 3 horses previously hyperimmunized for 2 years with California, Nevada and South Dakota virus and injected intracerebrally with Delaware and New Jersey virus 2 reacted severely and were sacrificed for humane reasons. One horse had a sharp febrile reaction and transitory symptoms of encephalitis but recovered. It had been previously recognized that the intracerebral mode of infection is an exceedingly drastic method to determine the immunity of horses against encephalitis (Meyer, Haring and Howitt, Records and Vawter), and doubtless not well suited for cross-immunity tests

(5) Twelve horses highly hyperimmunized for several months with Western viruses yielding a serum with antiviral substances injected intracutaneously with 1 or 2 cc and subsequently with 4 cc of a 20% suspension of Eastern virus survived. Several of the animals had slight transitory febrile reactions (39 to 40° C). Four control horses injected simultaneously by the same route developed typical encephalomyelitis, and either succumbed to the disease or were sacrificed

(6) Two horses hyperimmune to the Western virus and injected intracerebrally with Argentine virus showed no signs of illness. The control horse developed encephalitis and finally succumbed. During the febrile reaction the virus was demonstrated for from 12 to 60 hours in the blood serum. Simultaneously, horses hyperimmune to the California and Nevada virus were tested intracerebrally with the Utah virus. They failed to react

(7) One horse, which had survived an intracerebral injection of Argentine virus, tolerated the intracutaneous injection of the Eastern virus (4 cc)

(8) Two horses injected at weekly intervals with Eastern virus (1, 2 and 5 cc of a 20% virus suspension) failed to react to an intracerebral injection (5 cc) of a potent California virus, which fatally infected (with 2 cc.) a normal animal

## Susceptibility of Non-Immune, Hyperimmunized Horses and Goats to Eastern, Western and Argentine Virus of Equine Encephalomyelitis

K. F. MEYER, F. WOOD, C. M. HARING AND B. HOWITT

*From the Cutter Laboratory, the George Williams Hooper Foundation, and the Division of Veterinary Science, University of California, Berkeley and San Francisco, California*

Through the studies of TenBroeck and Merrill<sup>1</sup> and Giltner and Shahan<sup>2</sup> it is known that the Eastern viruses of equine encephalitis isolated during the epidemic of 1933 are serologically distinct from those responsible for the disease in California, Nevada, Colorado, Utah and South Dakota. Rosenbusch<sup>3</sup> studied a similar if not identical malady in Argentina. He found the South American virus immunologically identical with the California virus. A comparative study of the 3 viruses, on horses, suggested itself. The senior author is familiar with the clinical and pathological findings observed in horses during the 1912 epidemic in Delaware and during the outbreaks in California (1930 to 1933), Colorado and Texas. The results of these studies may be summarized as follows:

(1) Two Eastern viruses (Delaware isolated from a brain sent to California in glycerine, New Jersey courteously furnished by Dr. Carl TenBroeck) infected horses when injected intracerebrally, intravenously (filtrates) and intracutaneously. In one series 1 cc of a 20% suspension of guinea pig passage virus fatally infected 4 of 14 horses injected intracutaneously on the neck (distribution of cervical nerves). The clinical picture (fever, stupor and motor disturbances) differed in no way from that recorded in the many horses infected with the Western virus, although the course of the disease was greatly accelerated. Several animals died in from 62 to 163 hours after the administration of the virus or they were unable to rise between the 80th and 96th hour and were consequently sacrificed. Irrespective of the mode of infection, the virus was demonstrable in the blood serum from the 12th to 65th hours. The spinal fluid was invariably increased and turbid with a cell count of from 600 to 25 000 cells (5 to 20% granulocytes) and a positive Rivolta test and occasional web formation. The gross

<sup>1</sup> TenBroeck and Merrill, *Proc. Soc. Exp. Biol. and Med.*, 1933, **31**, 217

<sup>2</sup> Giltner and Shahan, *Science*, 1933, **78**, 587

<sup>3</sup> Rosenbusch, *Anales d. l. Sociedad Rural Argentina*, 1934, and personal communication to senior author

TABLE I  
 Chronological Appearance of the Virus of Equine Encephalomyelitis in the following tissues \*

Irr Serum	Nasal Mucosa	Maxillary and Sub										Anterior end of Forebrain
		Filtered	Cervical Glands	Olfactory Bulbs	Glands	Pons and Medulla	Cerebellum	Cornua Quadrigemina	Thalamus	Cerebral Hemispheres	Hind Brain	Mid brain
4	0	1st Series 0	0	0	0							0
8	+	2nd Series +	0	0	0						0	0
12		+	0	0	0						0	0
15		0	+	0	0						0	0
18	+	+	+	+	0						0	0
19	+	+	+	+	+						0	0
20	+	+	+	+	+						+	0
21	+	+	+	+	+	+		0	0	+	+	0
22	+	+	+	+	+	+		+	+	+	+	0
23	+	+	+	+	+	+		+	+	0	+	0
24	+	+	+	+	+	+		+	+	0	+	0
												{ 1st series 0 2nd series alt + }
36	+	0	+	+	+	+		+	+	+	+	+
48	+	+	+	+	+	+		+	+	+	+	+
96	+	+	+	+	+	+		+	+	+	+	+
120	0	+	+	+	0	+		+	+	+	+	+

\* + = virus present, 0 = no virus recovered

## 58 INTRANASAL INSTILLATION OF EQUINE ENCEPHALOMYELITIS

(9) The Eastern, Western and Argentine viruses injected intracerebrally may produce in goats a transitory or a rapidly fatal encephalitis

These observations indicate a very close relationship between the Western and Argentine virus not only with respect to infectivity but also with regard to cross protection. By contrast the Eastern virus of 1933 exhibits a greater virulence and thus may break the immunity established against the Western virus. On the other hand, the Eastern virus apparently protects against the Western virus. Further, these experiments lend considerable support to the conception of an insect transmission of the encephalitis virus as demonstrated by Kelser. The Eastern virus infects readily by the cutaneous route and the infective agent circulates for many hours in the blood of the horse.

7557 P

### Propagation of Virus of Equine Encephalomyelitis after Intranasal Instillation in the Guinea Pig

B F HOWITT (Introduced by K F Meyer)

*From the George Williams Hooper Foundation, University of California, San Francisco, California*

In an attempt to determine whether or not the virus of equine encephalomyelitis reported by Meyer, Haring and Howitt<sup>1</sup> spreads by way of an initial blood stream invasion and secondary penetration of the meningo-choroid plexus or by axonal propagation as in poliomyelitis,<sup>2,3</sup> the distribution of the infective agent in the tissues of guinea pigs following the intranasal instillation of the virus was studied.

Several series of small guinea pigs were each given 2 cc of a 20% saline suspension of California virus dropped into the nares. Three animals were killed by bleeding from the heart at each of the different periods of time as shown in Table I. No blood was removed at the twelfth hour, however. The tissues were removed

<sup>1</sup> Meyer, K F, Haring, C M., and Howitt, B, *Science*, 1931, 74, 227

<sup>2</sup> Faber, H K, and Gebhardt, L P, *J Exp Med*, 1933, 57, 933

<sup>3</sup> Flexner, S, *Science*, 1933, 77, 413

<sup>4</sup> Brodie, M., and Elvidge, A R., *Science*, 1934, 79, 235

<sup>5</sup> Schulze, E W, and Gebhardt, L P, *Proc. Soc. Exp. Biol. and Med.*, 1934,



Experimental Studies on *Trypanosoma cruzi* in California

FAE DONAT WOOD (Introduced by O. A. Kofoid.)

*From the Zoology Department and Hooper Foundation, University of California.*

The reduviid, *Triatoma protracta* Uhler, was reported to be a carrier of *Trypanosoma cruzi* Chagas, the haematozoon causing American human trypanosomiasis.<sup>1</sup> Examinations have revealed that the feces of 40 out of 73, or 54%, of a group of *Triatoma* from San Diego County were infected with *T. cruzi*. *Triatoma* from the vicinities of Berkeley and Los Angeles have not shown trypanosomes in their digestive tracts.

The San Diego wood rat has been incriminated as a reservoir host of *T. cruzi*. Of 43 rats examined only one was infected. This was a light infection and was detected by examination of centrifuged, citrated heart blood. The trypanosome infecting this rat was identical in morphology and behavior with that found in animals infected experimentally from *Triatoma* feces, and also with a known strain of *T. cruzi* from Professor Brumpt's Paris laboratory.

The southern parasitic mouse, the San Diego desert mouse, and the Virginia opossum, all associated with San Diego wood rats in nature, are more susceptible to the infection in the laboratory than the rats themselves, so it is possible that they, too, may be natural carriers of this trypanosome.

Portola wood rats, from Berkeley, harbor a trypanosome of the "lewisii" type which should not be confused with *T. cruzi*.

One hundred thirty-four animals, including 16 species, have been inoculated with the California strain of *T. cruzi*. The following list gives the species and number of animals inoculated, the number in parenthesis indicating how many animals became infected: 53 (30) albino Norway rats, 23 (7) albino mice, 2 (1) puppies, 2 (1) Virginia opossums, 4 (3) rhesus monkeys, 12 (7) San Diego wood rats, 3 (1) Portola wood rats, 5 species of white-footed mice [4 (4) parasitic, 9 (8) southern parasitic, 3 (3) San Diego desert, 5 (1) Gambel, 3 (1) Gilbert], 2 (0) rabbits, 6 (0) guinea pigs, 3 (0) kittens, and 1 (0) desert antelope ground squirrel.

Infection has been produced by the following methods of inoculation: infective *Triatoma* feces intraperitoneally, subcutaneously, intramuscularly, in the eyes, mouth, or on scarified skin, citrated

<sup>1</sup> Kofoid, C. A., and Donat, F., *PROG. SOC. EXP. BIOL. AND MED.*, 1933, **30**, 489.

aseptically, pooled from the 3 animals, ground with saline and injected intracranially into guinea pigs. The nasal mucosa was extracted in saline and passed through a Seitz filter before inoculation. The serum was given both into the brain and subcutaneously. Several guinea pigs were allowed to live until prostration as controls on the viability of the virus. They all succumbed to the disease.

The results as given in Table I show an immediate and earlier invasion of the blood stream for this experiment than that previously reported<sup>6</sup> for guinea pigs. The virus was constantly present in the serum after the eighth hour and was almost always recovered from the nasal mucosa. Its constant presence in this tissue, however, may have been due to the amount of blood necessarily obtained from this region. It was then recovered from the cervical and salivary glands, the olfactory bulbs and subsequently from the cerebral nerve tissues beginning with the hind brain. After the 36th hour, coincident with the rise in temperature, the virus was constantly present in all of the tissues examined, except for the disappearance from the blood after defervescence and the subsequent prostration of the animal.

To further corroborate the evidence just given of the primary invasion of the blood stream rather than the nerve tissues, an experiment similar to that described by Brodie<sup>4</sup> and by Schulze and Gebhardt<sup>5</sup> for poliomyelitis in monkeys was performed on guinea pigs. The olfactory bulbs were removed surgically from 4 guinea pigs under anesthesia. After one week they, together with 4 normal animals were given 2 cc. of virus intranasally. All 8 guinea pigs succumbed to the disease with typical symptoms. To show that the virus had not been absorbed through the digestive tract, 2 more animals were fed 2 cc. each of the same virus by catheter into the stomach. Neither of them developed the disease.

From the evidence obtained it would seem that the virus of equine encephalomyelitis when given intranasally, gains entrance primarily into the blood stream, presumably through the vascular nasal mucosa and that there is a systemic septicemic invasion before localization in the nerve tissue. A more detailed report of this study will be given later.

<sup>6</sup> Howatt, B. F., *J. Infect. Dis.*, 1932, **51**, 493

## Inhibition by Glucose of Methemoglobin Formation

MATILDA MOLDENHAUER BROOKS

*From the Department of Zoology, University of California, Berkeley*

The purpose of these experiments is to show that injection of glucose prevents the formation of methemoglobin in animals, or if methemoglobin is already present, it can by the same means be reduced to hemoglobin which is then readily oxygenated to oxyhemoglobin

Methemoglobin was produced experimentally in rabbits by injections of 0.15 gm of  $\text{NaNO}_2$  for every kg of body weight. This dose converted 15% of the blood-hemoglobin into methemoglobin in a few minutes. This is less than the theoretical effect predicted by Wendel<sup>1</sup> for this dose, owing no doubt to the constant presence of glucose normally in the blood stream. Glucose was used in amounts of 1 cc to 2 cc of a 1% solution for every kg body weight. All solutions were made up fresh with 0.9% NaCl and injected intravenously into one ear of the rabbit, blood samples were taken from the other ear at intervals thereafter.

The spectrophotometric method was used for determining the proportions of methemoglobin in blood. This method is sensitive to less than 2%. The ratio,  $R$ , of the extinction coefficient at  $\lambda = 540 \text{ m}\mu$  to that at  $\lambda = 560 \text{ m}\mu$ , as found by Ray, Blair and Thomas<sup>2</sup> indicates the per cent of methemoglobin present. All blood samples were diluted to 1% with 0.4%  $\text{NH}_4\text{OH}$ . No difference in readings was noted when water was used as a diluent. The thickness of the layer measured was 1 cm. Five animals were used in each group. The probable error of the readings was a fraction of 1%. The method is also valuable because determinations can be made so quickly that chemical changes following collection are minimized.

**Results *in vivo*** Methemoglobin was produced in the rabbit as indicated above, the  $R$  value found being 1.56 which indicates 15% conversion to methemoglobin. Glucose was then injected. Five minutes later the  $R$  value was 1.66 which indicates complete reconversion of methemoglobin to oxyhemoglobin. The controls which had not received glucose still showed 15% methemoglobin present. If glucose is injected before the nitrite, no methemoglobin can be demonstrated even after several hours. Injections of saline in the

<sup>1</sup> Ray, G. B., Blair, H. A., and Thomas, C. L., *J. B. C.*, 1932, 98, 63.

<sup>2</sup> Wendel, W. B., *J. Am. Med. Assn.*, 1933, 100, 1054.

blood intraperitoneally, subcutaneously, or intramuscularly, and culture forms intraperitoneally

Groups of leishmaniform bodies have been found in bone marrow and cardiac and voluntary muscles of infected animals. Lesions composed of infiltrating lymphocytes, monocytes, and plasma cells were seen in cardiac and voluntary muscles, cerebrum, and meninges. Some cases have shown fatty degeneration of the liver.

The parasites, both in the blood and in the tissues, have been very scarce in most cases. The only animal to show any symptoms, namely retarded growth and the temporary paralysis of the hind legs, was the southern parasitic mouse. In no case was the disease fatal.

Attempts were made to intensify the infection by lowering the host's resistance by splenectomy, by injection of testicle extract, and by keeping the animals at a higher temperature.

Previously<sup>1</sup> splenectomy seemed to stimulate the appearance of *T. cruzi* in latent infections. Further experiments with a larger number of animals indicate that splenectomy has no real effect. Eight (57%) out of 14 splenectomized, and 22 (56%) out of 39 non-splenectomized, albino rats became infected after inoculation. The difference of 1% can hardly be considered significant.

Duran-Reynals<sup>2</sup> reported that injection of testicle extract increased the invasiveness of a neurovirus in rabbits. Experiments indicate that testicle extract has no such stimulating effect upon *T. cruzi*.

Six albino mice placed in an incubator at 34 to 36° C did not take heavier infections than controls kept at room temperature.

Successive passages through different host species (puppy, albino rat, San Diego wood rat, albino rat), covering a period of 103 days indicated a stimulating effect upon the trypanosomes in that the incubation period progressively decreased (35, 26, 22, 20 days). In 2 control experiments in which the trypanosome was passed through animals of the same species, *i. e.*, young albino rats or mice, the parasites failed to appear after the eighth and fourth passages, respectively.

*T. cruzi* has been successfully cultured and subcultured on semi-solid blood-agar. Material from the original cultures and from the first subcultures has produced typical infections in white-footed mice.

<sup>2</sup> Duran Reynals, F, *J. Exp. Med.*, 1929, 50, 327

growing tips of several dozen stems were cut off and kept in a large beaker of pond water in the dark for 24 hours prior to use. These clusters of cells were from 2 to 10 mm in diameter.

The manometric technique, originally described by Barcroft and Haldane, and subsequently employed extensively by Warburg, was used for measuring oxygen consumption. The material was immersed in pond water. Five per cent KOH was used in the inset to absorb  $\text{CO}_2$ , and the vessels containing the green plants were wrapped in an opaque black cloth to exclude light. Oxygen consumption was measured as a rate,  $Q_{\text{O}_2}$ , in cmm per hour per gram wet weight.

The effects of NaCN and methylene blue, singly and combined were studied, purely relative results being obtained when the agents were used separately. When methylene blue was added after the cyanide, all values were corrected for the percentage change of a control run at the same time. In every case the normal  $Q_{\text{O}_2}$  was established for the second hour of a 2-hour run. In the case of cyanide the vessels were then disconnected and 0.4 cc of the proper NaCN concentration was added, and the  $Q_{\text{O}_2}$  of the second hour thereafter determined. When methylene blue was used alone the dye was poured into the plant environment from the side-arm after the normal  $Q_{\text{O}_2}$  had been established. When methylene blue was used in combination with NaCN, the percentage inhibition was first determined with cyanide alone, the methylene blue then added and the  $Q_{\text{O}_2}$  determined for the second hour thereafter, in conformance with the standard procedure. All inhibition and acceleration were expressed as percentage deviation from the normal as first established in each case.

The results are given in Table I, negative indicating the percentage decrease in oxygen consumption, and positive the increase.

TABLE I

Concentration	Effects of NaCN Alone					Effects of Methylene Blue Alone			
	10-1M	10-2M	10-3M	10-4M	10-5M	10-2M	10-3M	10-4M	10-5M
% deviation	-6%	-44	-46, -51	-29	-12	+107	+74	+43	+5
from normal	+35	-33	-42, -44	-20	-8	+123	+66	+48	-1
Aver	+8	-39	-46	-25	-10	+115	+70	+46	+2

Since the cells were definitely and irreversibly injured in  $10^{-1}$  M cyanide, the absence of inhibition is probably due to injury. Injury probably also decreases inhibition by  $10^{-2}$  M cyanide, though not to so great an extent. Normal oxygen consumption was resumed

place of glucose have no effect on methemoglobin formation or its change to oxyhemoglobin

Results *in vitro* with sheep blood also showed that methemoglobin formation is delayed if glucose is present, or if already formed, the addition of glucose will reconvert a part of it to oxyhemoglobin. The following experiment will illustrate this: to 4 cc of washed r b c suspended in 0.9% saline solution, there was added 0.5 cc of M/20 phenylhydroxylamine freshly prepared. R was then found to be 1.42, indicating 48% methemoglobin. This solution was divided into 2 parts: glucose was added to one part, and to the other part only saline. The glucose-containing blood now had only 18% methemoglobin present ( $R = 1.53$ ) while that with a saline alone remained unchanged at 48% ( $R = 1.42$ ). It has not been found possible to reduce *in vitro* all the methemoglobin to oxyhemoglobin. This was also found by Warburg, Kubowitz and Christian,<sup>3</sup> who state that unknown reactions take place.

Similar effects can be demonstrated using  $\text{NaNO}_2$  instead of phenylhydroxylamine. If glucose be added before the methemoglobin-producing substances, the formation of methemoglobin is delayed and the sample is still reddish when the control is brown.

These results show that glucose is effective in preventing methemoglobin formation, or after formation, in reducing it to hemoglobin which can then form oxyhemoglobin. It is suggested that the presence of glucose in the blood stream is responsible for the often observed failure of various agents to produce the expected proportion of methemoglobin. It is also suggested that injections of glucose be used clinically in cases where methemoglobin is present either as a result of pathological conditions or as a result of poisoning by such methemoglobin-producing substances as aniline dyes, nitrites, etc.

## 7560 P

### Action of Respiratory Catalysts and Inhibitors on Oxygen Consumption by Nitella.

EDWARD ROSS (Introduced by S. C. Brooks)

From the Department of Zoology, University of California

The material used consisted of young, actively growing coenocytic cells of *Nitella clavata* collected from a large outdoor pool. The

<sup>3</sup> Warburg, O., Kubowitz, F., and Christian, W., *Biochem. Z.*, 1931, **242**, 170

growing tips of several dozen stems were cut off and kept in a large beaker of pond water in the dark for 24 hours prior to use. These clusters of cells were from 2 to 10 mm. in diameter.

The manometric technique, originally described by Barcroft and Haldane, and subsequently employed extensively by Warburg, was used for measuring oxygen consumption. The material was immersed in pond water. Five per cent KOH was used in the inset to absorb  $\text{CO}_2$ , and the vessels containing the green plants were wrapped in an opaque black cloth to exclude light. Oxygen consumption was measured as a rate,  $Q_{\text{O}_2}$ , in cmm per hour per gram wet weight.

The effects of NaCN and methylene blue, singly and combined were studied, purely relative results being obtained when the agents were used separately. When methylene blue was added after the cyanide, all values were corrected for the percentage change of 1 control run at the same time. In every case the normal  $Q_{\text{O}_2}$  was established for the second hour of a 2-hour run. In the case of cyanide the vessels were then disconnected and 0.4 cc of the proper NaCN concentration was added, and the  $Q_{\text{O}_2}$  of the second hour thereafter determined. When methylene blue was used alone the dye was poured into the plant environment from the side-arm after the normal  $Q_{\text{O}_2}$  had been established. When methylene blue was used in combination with NaCN, the percentage inhibition was first determined with cyanide alone, the methylene blue then added and the  $Q_{\text{O}_2}$  determined for the second hour thereafter, in conformance with the standard procedure. All inhibition and acceleration were expressed as percentage deviation from the normal as first established in each case.

The results are given in Table I, negative indicating the percentage decrease in oxygen consumption, and positive the increase.

TABLE I.

Concentration	Effects of NaCN Alone						Effects of Methylene Blue Alone			
	10 <sup>-1</sup> M	10 <sup>-2</sup> M	10 <sup>-3</sup> M	10 <sup>-4</sup> M	10 <sup>-5</sup> M		10 <sup>-2</sup> M	10 <sup>-3</sup> M	10 <sup>-4</sup> M	10 <sup>-5</sup> M
% deviation	-6%	-44	-46, -51	-29	-12		+107	+74	+43	+5
from normal	-5	-33	-42, -44	-20	-8		+123	+66	+48	-1
Aver	+35		-44, -51							
	+8	-39	-46	-25	-10		+115	+70	+46	+2

Since the cells were definitely and irreversibly injured in  $10^{-1}$  M cyanide, the absence of inhibition is probably due to injury. Injury probably also decreases inhibition by  $10^{-2}$  M cyanide, though not to so great an extent. Normal oxygen consumption was resumed

when  $10^{-3}$  M cyanide had been washed out. There was, therefore, no irreversible injury at this or lower concentrations. Acceleration was perfectly reversible on washing out the methylene blue taken up from  $10^{-4}$  M solution, but after  $10^{-3}$  M it was not possible to wash out all the dye within a reasonable time. Injury cannot be surely excluded in the case of  $10^{-2}$  M solution of the dye.

The antagonism between cyanide and methylene blue has been tested by 3 experiments so far. In all of these  $10^{-3}$  M NaCN was used. The results, corrected for percentage changes in the controls, are given in Table II.

TABLE II.  
Effects of Methylene Blue Added after Cyanide

Experiment	1	2	3
Effect of NaCN, $10^{-3}$ M	-44%	-33%	-36%
Concentration of dye added	$10^{-3}$ M	$10^{-3}$ M	$10^{-4}$ M
Resultant effect (0.0%—complete recovery)	-9%	-41%	-39%

The dye appears to have accelerating power only when its concentration is greater than that of the inhibitor. With this material the apparent acceleration may have been due to injury, investigation of which is in progress.



# New York Meeting

*New York Academy of Medicine, October 17, 1934*

7561 C

## Further Experiments on the Effect of Testicle Extract on the Agent of Chicken Tumor I

F DURAN REYNALS AND ALBERT CLAUDE (Introduced by J B Murphy)

*From the Rockefeller Institute for Medical Research, New York*

Hoffman, Parker, and Walker<sup>1</sup> reported that rabbit testicle extract markedly enhanced the growth of Chicken Tumor I, the effect being the same whether tumor mash or cell-free filtrates of the tumor were used in the inoculations Sturm and one of us<sup>2</sup> could not duplicate these findings despite the use of a variety of both testicle extracts and active tumor preparations

Since more work was indispensable in order to elucidate the cause of the discrepancies we first resorted to the technique of progressive dilution of the active tumor material Accordingly, water dilutions of the fresh tumor tissue, ranging from 1 6 to 1 10,000 were obtained and 0 5 cc of each dilution was injected with its volume of filtered bull testicle extract diluted 1 2 Each chicken was inoculated intradermally in 2 or 3 areas in each side of the

TABLE I

No of tests	Dilution of tumor extracts	Aver area of tumor surface after 14 days		Aver area of tumor surface after 21 days	
		Testicle extract	H <sub>2</sub> O	Testicle extract	H <sub>2</sub> O
		sq cm	sq cm.	sq cm.	sq cm.
9	1 6	6.2	4.8	12.0	8.7
8	1 60	4.0	4.1	6.1	9.1
8	1 800	1.6	1.1	4.7	3.4
3	1 600	4.5	1	5	0
2	1 3,000	0.5	0	1	0
1	1 12,000	0	0	3.0	0
1	1 30,000	0	0	2.0	0
1	1 60,000	0	0	0	0

<sup>1</sup> Hoffman, D C, Parker, F, and Walker, T T, *Am J Path*, 1931, 7, 523

<sup>2</sup> Sturm, E, and Duran Reynals, *J Exp Med*, 1932, 56, 711

breast The customary spreading was noticed after each injection The results are summarized in Table I

Table I shows some enhancement by testicle extract, but far less regular and marked than in the case of viruses

In the foregoing tests, the mixture was inoculated intradermally at points located on both sides of the median breast line With the bird in normal position this region is more or less horizontal and occupies the lowest part of the body It is obvious that in this case the mixture injected had little opportunity to spread Under special conditions the spreading agent may even drain back the active material This fact may well account for the observation that in certain cases tumors obtained from testicle extract mixtures were smaller than the controls

In the following experiments, the material was inoculated into the skin, either in a lateral region under the wing, or in the upper part of the leg The active material was obtained by extracting fresh chicken tumor pulp with 12 volumes of sterile distilled water The fluid after centrifugation was used for the test and mixed before inoculation with an equal volume of fresh rat testicle extract The latter had been prepared in the usual manner by extracting with an equal volume of water, and using the supernatant fluid from centrifugation

As illustrated in Table II, the results were conclusive and the tumors obtained were incomparably larger than in the case of the control injection, simply diluted with an equal volume of Ringer's solution

TABLE II.

Exp No	No of tests	Amt. of mixture injected	Aver size of main tumor surface after 18 days		Aver area of skin with scattered nodules around the main tumor	
			Testicle extract	Ringer's solution	Testicle extract	Ringer's solution
		cc.	sq cm	sq cm.	sq cm.	sq cm.
1	1	5	32.8	8.6	51.1	0
2	1	5	12.0	8.6	36.0	0
3	2	8	26.7	3.7	59.0	0
4	2	8	19.5	3.7	40.8	0
5	3	6	61.2	7.0	0	0
6	3	6	46.0	6.3	0	0
7	1	6	32.0	5.0	0	0
Average surface of tumor			34.0	6.1	48.4	0

*Conclusion* In agreement with the results of Hoffman, Parker, and Walker, Chicken Tumor I agent is spread when injected together with testicle extract and the resultant lesions are markedly enhanced

## 7562 P

## Protective Substances in Sera of Animals Injected with Anterior Pituitary-Like Hormone of Teratoma Testis Urine

GRAY H. TWOMBLY AND RUSSELL S. FERGUSON (Introduced by J. Ewing)

*From the Memorial Hospital, New York City*

Collip<sup>1</sup> reported observations on the results of repeated inoculations of the thyreotropic hormone, in large amounts, over prolonged periods of time into white rats. He showed that, on injection of the thyreotropic principle of the pituitary, hyperplasia of the thyroid gland occurs and the metabolic rate rises sharply. However, continued injections fail to maintain this condition and the metabolic rate returns to normal in from 2 to 3 weeks and may even become subnormal. The animals fail to show any rise in metabolism on the administration of as much as 8 times the previously effective dose. The serum of animals, thus rendered refractory, will exert a protective effect against the activity of the hormone when injected into untreated animals. It does not protect, however, from the action of desiccated thyroid tissue. Anderson and Collip<sup>2</sup> have prepared a potent antithyreotropic serum by repeated injections into a horse. Similar "antihormones" inhibiting the action of the anterior pituitary-like hormone of pregnancy urine have been made and reported by Selye, Bachman, Thompson, and Collip.<sup>3</sup>

In an attempt to confirm this work and to determine whether the same thing is true of the anterior pituitary-like hormone found in the urine of patients suffering from teratoma testis, we have injected a number of rabbits over prolonged periods of time. The anterior pituitary-like hormone was obtained according to the original method of Zondek<sup>4</sup> by precipitation of acidified fresh urine with 5 times its volume of 95% ethyl alcohol. The precipitate was washed 3 times with ether, dried, and stored in the ice box. Due to the difficulty of obtaining large amounts of the urine, the extracts were prepared from lots of 300 to 1000 cc. These were assayed separately for their potency by the injection of watery extracts into immature female mice. Most of the urine came from 2 patients and was found to contain 5,000 to 10,000 mouse units per liter.

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<sup>1</sup> Collip, J. B., *J. Mount Sinai Hosp.*, 1934, 1, 28.

Anderson, Evelyn M., and Collip, J. B., *Lancet*, 1934, 1, 784.

<sup>3</sup> Selye, Hans, Bachman, C., Thompson, D. L., and Collip, J. B., *Proc. Soc. Exp. Biol. and Med.*, 1934, 31, 1113.

<sup>4</sup> Zondek, Bernhard, *Die Hormone des Ovariums und des Hypophysenvorderlappens*, Berlin, Julius Springer, 1931.

Three litter-mate female rabbits 13 weeks old, weighing 2 kilos each, were injected with approximately 100 mouse units of an aqueous extract of the powder containing the hormone, daily for a period of  $3\frac{1}{2}$  months. Two days after cessation of the injections the animals were bled and the serum separated. This serum was tested for its protective effect against the action of the original hormone in the following fashion. Infantile female mice, weighing 6 to 8 gm were injected with approximately 4 mouse units of the aqueous solution of the hormone and 0.5 cc of the rabbit serum, over a period of 30 hours (0.2 cc of hormone solution and 0.1 cc of serum given subcutaneously at 12 a. m., 4 p. m., 9 a. m., 12 a. m., and 4 p. m.). One hundred hours after the beginning of the injections the animals were killed and their ovaries inspected according to the usual technique of the Aschheim-Zondek test. Thirty-nine mice so treated failed to show any corpora lutea while 24 mice receiving the extract alone all showed large corpora lutea and open vaginal orifices. Thirteen mice were given the aqueous extract plus the serum of normal rabbits. The serum was obtained from 5 different animals. All the mice so treated showed corpora lutea, that is, the sera of these rabbits failed to show any protective effect against the action of the hormone such as was exhibited by the sera of the injected rabbits.

The injection of only 0.3 cc. of serum into each mouse also protected against the action of the hormone. Three mice injected with 0.1 cc only, showed protection in one, no protection in one, and apparently no corpora lutea but an open vaginal orifice in the third. That is, there was sufficient protective substance in 0.3 cc of the sera of the injected rabbits to counteract completely the action of 4 mouse units of A P L from teratoma urine. The experiments were clear cut, none of the mice receiving half a cc of immune serum showing corpora lutea and all of the controls showing luteinization.

A similar experiment was tried with the preparation of the A P L hormone of pregnancy urine made by E. R. Squibb and Sons, called by them "Follutein". This extract in glycerine contained 250 mouse units per cc and was said to have 0.55 mg of N per cc. The serum of 3 female rabbits, weighing approximately 2 kilos each, was tested for any protective effect it might show against the action of the hormone, each mouse being given 0.5 cc of serum and 5 mouse units of Follutein. Eleven mice so tested failed to show any evidence of protective substance. The rabbits were then injected daily with 75 mouse units (375 rat units) and the injections continued, with occasional interruptions, for a month. At the end of this

time samples of the serum all showed complete protective action in 0.5 cc. amounts against 5 mouse units of Follutein. The injections were continued for another 2 weeks, at the end of which time the rabbits showed multiple sores in the skin of the back, a phenomenon which had been previously noted in the animals injected with A P L from teratoma testis urine. All these animals continued to show a protective effect in their serum. A male rabbit injected for a month gave similar results. Twenty-seven mice tested with the sera after injection showed complete protection. Twelve control mice receiving Follutein alone all showed corpora lutea.

Cross protection experiments of this serum and the teratoma testis urine hormone were carried out. Three mice injected with the teratoma hormone alone showed corpora lutea while 9 receiving 4 mouse units of the hormone plus 0.5 cc. of serum immunized against the pregnancy hormone each showed no effect in the ovaries. Nine mice receiving four mouse units of "Follutein" plus 0.5 cc. of serum from the rabbits immunized against the teratoma hormone showed no evidence of luteinization while three control mice injected with "Follutein" alone showed corpora lutea.

### 7563 P

#### Successive Transmission of Virus of Lymphogranuloma Inguinale Through White Mice

ARTHUR W. GRACE AND FLORENCE H. SUSKIND

(Introduced by Paul Reznikoff)

*From the New York Hospital and Department of Medicine, Cornell University Medical College, New York*

As part of a study of the properties of the virus of lymphogranuloma inguinale white mice were inoculated intracerebrally with bacteriologically sterile pus aspirated from an inguinal bubo and glandular material obtained from a case of lymphogranuloma inguinale. The pus and glandular material were diluted 1 in 5 with sterile distilled water and inoculated in 0.03 cc. quantities into each of 6 mice. The object of this section of the work was to ascertain whether or not the virus could be transmitted indefinitely in that manner. All of the inoculated animals died within an average of 11 days. The brain of one of these animals dying from lymphogranuloma inguinale was emulsified in 1 in 2.5 dilution of distilled water and inoculated intracerebrally in 0.03 cc. quantities into another batch of 6 white mice. All of these mice died within an

average of 70 days. A similar procedure caused the death of the next four generations of mice in an average of 54 days and by the time the eleventh generation was reached, the animals were expiring in four days. Similarly, there was an increased mortality rate with successive passage, 90 to 100% of all the inoculated animals succumbing in the seventh to the eleventh generations. The shortening of the period taken for the mice to die involved such frequent subpassage that the strength of the inocula of subsequent generations was reduced to a 1 in 5 dilution. The effect of the increased dilution was to make the average time taken for the mice to die longer and to decrease the mortality rate. Nevertheless, there continued to be an increase in the virulence of the virus so that by the 26th and 27th generations, when a 1 in 25 dilution was again employed, 100% of all the inoculated animals died in an average of 26 days. At the time of writing (35th generation) 80 to 100% of mice die in an average of 67 days as a result of an intracerebral inoculation of a 1 in 5 suspension of lymphogranulomatous mouse brain. Physiological saline, Tyrode's solution, ascitic fluid or infusion broth of pH 8.0 are diluents equally as good as sterile distilled water.

A control series of inoculations in which normal mouse brain was used instead of lymphogranulomatous mouse brain, did not produce any effect in mice.

Evidence of the presence of the virus of lymphogranuloma inguinale in the brains of the dead mice was furnished by the production of highly potent Frei antigens from these brains, the method of preparation of the mouse brain antigen was identical with that used for the preparation of Frei antigens from human lymphogranulomatous pus. The potency of the antigen appears to increase with successive passage of the virus. Normal mouse brains prepared and tested as Frei antigens do not produce any appreciable reactions.

Histological examination of the brains of the dead mice indicates that the virus produces a meningitis in which the predominant cell is of the lymphocyte type, polymorphonuclear leucocytes are comparatively sparse.

*Conclusion* One strain of the virus of lymphogranuloma inguinale, upon intracerebral inoculation into white mice, has quickly developed a fixed virulence for these animals and appears to be capable of transmission indefinitely in this manner.

Frei antigens prepared from lymphogranulomatous mouse brains are specific and highly potent, the antigenic strength increases with successive mouse passage.

## 7564 P

## Renal Excretion of Inulin, Creatinine and Xylose in Normal Dogs

A. N. RICHARDS, B. B. WESTFALL AND PHYLLIS A. BOTT

*From the Laboratory of Pharmacology, University of Pennsylvania.*

During the course of studies of the properties of the renal tubule of amphibia we had occasion to search for a substance which, introduced into the tubule, would not be expected to be absorbed from it into the blood, either actively or by diffusion. Among those tried was the polysaccharide inulin, chosen because of its high molecular weight and because it is not hydrolyzed by enzymes or tissues of vertebrates. After having found that inulin is much less rapidly diffusible than either creatinine or glucose, that it is filterable through collodion membranes which are impermeable to protein and through the glomerular membranes of amphibia, and also that it is not excreted by the aglomerular kidney (toadfish) after intravenous injection, it seemed desirable to study its rate of excretion in mammals. Given intravenously to dogs or rabbits it is excreted in the urine rapidly, and, insofar as a few experiments show, completely. Concentration ratios, U/P, as high as 150 have been observed. It became obvious that some of the considerations developed by Jolliffe, Shannon and Smith<sup>1</sup> upon which they based their advocacy of plasma clearance of xylose in preference to that of creatinine as a true measure of the volume of glomerular filtration could equally well be made the basis of similar advocacy of inulin clearance for the same purpose, with this advantage, that the low diffusibility of inulin, much lower than that of creatinine, of xylose and of the other 2 non-metabolized sugars tested by them (sucrose, raffinose) could be expected to minimize a difference between plasma clearance and rate of glomerular filtration, provided such a difference exists and is the result of back diffusion of the substance studied. Accordingly, a series of preliminary experiments has been made, admittedly not perfect, in which the simultaneous plasma clearances of inulin and creatinine have been determined in unanesthetized female dogs. The results, which seem sufficiently convincing to put on record, indicate that the plasma clearance of inulin given intravenously is slightly higher than, but of the same order as that of creatinine.

<sup>1</sup> Jolliffe, N., Shannon, J. A., and Smith, Homer W., *Am. J. Physiol.*, 1932, 100, 301, 102, 534.

A year later a similar series was made in which simultaneous clearances of inulin and xylose were determined. The results indicate that inulin clearance is significantly higher than that of xylose.

Inulin was given intravenously 1 to 4 gm before beginning the experiment, slow continuous infusion during it. Creatinine was given intraperitoneally, xylose by mouth.

Urine collection periods varied from 12 to 52 minutes, average, 27. Blood from the jugular vein was taken one minute before the beginning and one minute before the end of each period. Urine was collected by catheter.

Inulin and xylose in plasma and urine were determined by the Shaffer-Somogyi method<sup>2</sup> applied to the properly diluted fluids (a) before hydrolysis, (glucose + xylose), (b) before hydrolysis, after fermentation with washed yeast (xylose), (c) after hydrolysis with 0.1 N HCl (glucose + xylose + inulin). Urines were diluted to make the reducing powers approximately the same as those of the corresponding plasmas.

Creatinine was determined by Folin's method as used by Holten and Rehberg.<sup>3</sup>

Twenty-three comparisons of inulin and creatinine clearances were obtained in 10 experiments on 5 normal dogs in June and July, 1933, 11 more in 5 experiments on 4 dogs in July, 1934.

Twenty-five comparisons of inulin and xylose clearances were made in 11 experiments on 4 normal dogs in May, June and July, 1934. In any single experiment clearance of only 2 of the 3 substances was measured.

Average plasma concentrations of inulin ranged from 60 to 346, of creatinine, from 3.3 to 26.0, of xylose, from 30 to 233 mg %. Rates of urine excretion varied from 0.44 to 5.8 cc per min.

The results, given as clearance ratios, were as follows:

A *Inulin clearance/Creatinine clearance* 0.64, 0.64, 0.73, 0.84, 0.85, 0.87, 0.92, 0.92, 0.96, 0.97, 1.00, 1.00, 1.01, 1.02, 1.03, 1.03, 1.05, 1.05, 1.08, 1.11, 1.11, 1.14, 1.14, 1.14, 1.15, 1.16, 1.18, 1.20, 1.20, 1.21, 1.36, 1.38, 1.46, 1.53. Mean, 1.06.

B *Inulin clearance/Xylose clearance* 0.85, 0.89, 0.93, 1.03, 1.08, 1.09, 1.11, 1.16, 1.16, 1.16, 1.23, 1.25, 1.27, 1.33, 1.35, 1.35, 1.39, 1.41, 1.43, 1.47, 1.49, 1.49, 1.59, 1.59, 1.67. Mean, 1.27.

The difference between the 2 groups of results seems significant.

No correlation was found between inulin clearance and plasma concentration of inulin or rate of urine flow.

<sup>2</sup> Shaffer, P. A., and Somogyi, M., *J. Biol. Chem.*, 1933, **100**, 695.

<sup>3</sup> Holten, C., and Rehberg, P. B., *Acta Med. Scand.*, 1931, **74**, 479.



The concentrations of inulin in the urine were astonishing in one experiment 35.8%, in another 32.3, in another 22.4. In one instance the concentration ratio, U/P, was 150, in another 132.

It must be stated that in more than half the experiments undeniably large variations in the plasma concentrations of the substances studied (particularly of inulin) occurred, due presumably to the methods of administration. Exclusion of these from consideration does not, however, alter the conclusion drawn from the series as a whole, *viz.*, that the plasma clearance of injected inulin is of the same order as that of injected creatinine and higher than that of xylose. We are inclined to ascribe this difference to greater diffusion of xylose than of inulin from the renal tubule.

One main purpose of this publication is to call attention to the possible usefulness of inulin in connection with renal studies.

## 7565 C

### Occurrence of Non-Motile Leucocytes

M. SCHWEIZER. (Introduced by Eric Ponder.)

*From Washington Square College, New York University*

Sabin, Cunningham, Doan and Kindwall<sup>1</sup> observed that when supravital counts were made on blood drawn every 15 minutes there appeared "showers of non-motile cells" at approximately hourly intervals. They interpreted these as being due to degenerating polymorphs which were actually dying in the blood stream. Beard and Beard<sup>2</sup> confirmed the existence of these showers, and were able to increase their magnitude without altering their rhythm by the injection of sodium citrate. More recent workers (Smith and McDowell,<sup>3</sup> Jones, Stephens, Todd, and Lawrence<sup>4</sup>) have been unable to confirm these observations, and find that the non-motile cells occur at irregular intervals and are probably artefacts.

If the non-motile cells are really dying polymorphs, most of them should be old cells, as determined by the criterion of the number of lobes in the nucleus (Cooke and Ponder<sup>5</sup>), whereas if they are

<sup>1</sup> Sabin, Cunningham, Doan and Kindwall, *Johns Hopkins Hosp. Bull.*, 1925, **37**, 14.

<sup>2</sup> Beard and Beard, *Proc. Soc. Exp. Biol. and Med.*, 1927, **24**, 614.

<sup>3</sup> Smith and McDowell, *Arch. Int. Med.*, 1929, **43**, 68.

<sup>4</sup> Jones, Stephens, Todd and Lawrence, *Am. J. Physiol.*, 1933, **105**, 547.

<sup>5</sup> Cooke and Ponder, *The Polynuclear Count*, 1927, London.

artefacts, we might well expect both young and old cells to be affected, i.e., cells of all the classes of the polynuclear count. I have made a series of counts to determine (a) whether the non-motile cells occur regularly, and (b) whether they are always old cells, i.e., cells of the higher classes of the polynuclear count. For each count 2 preparations were made under separate coverslips but on the same slide, and these were examined by the supravital technique, counts of non-motile cells being made in regions of the preparations which initially showed many motile forms. Table I shows a typical series of results. The preparation designated by (a) was the one counted first.

TABLE I

Preparation	Total cells counted	Polymorphs, %	Non motiles, %
1 a	100	72	12
1 b	100	73	4
2 a	100	73	1
2 b	150	67	9
3 a	150	74	8
3 b	200	74	3
4 a	100	73	2
4 b	150	76	14
5 a	200	75	17
5 b	200	73	4

The nuclei of the non-motile cells became swollen, and the lobation is often obscured. It is possible, in fact, to count the nuclear lobes in only about half the cases. The data on this point are, in consequence, purely qualitative, but where it was possible to distinguish the number of nuclear lobes it was found that there were generally 2 lobes, sometimes one or 3 lobes, and only once 4 lobes. Taken together, the results show that the percentage of non-motile cells show large fluctuations even under the same conditions (i.e., there are large differences between the percentage of these cells in 2 preparations made at the same time from the same sample of blood), whereas the percentage of total polymorphs remains very constant, and also that the non-motile cells are not necessarily old cells. It is a little surprising that a larger number of polymorphs of class IV were not seen, but these, the oldest cells of all, may well have been represented among the non-motiles whose nuclear lobation was impossible to determine. It is to be concluded that the non-motile cells are produced by some uncontrollable factor involved in making the preparation, and that the presence of these cells can not be interpreted as indicating the presence of dying polymorphs in the blood stream.

From purely numerical considerations, it is difficult to see how

one could expect to detect the presence of dying cells by any of the known methods of counting even if they were to die in "showers" at hourly intervals. Assuming that there are 7000 polymorphs per  $\text{mm}^3$  of blood, and that the average life of these cells is about 15 days (Cooke and Ponder<sup>5</sup>), there would be 20 cells per  $\text{mm}^3$  of blood dying each hour, or only 0.002% of the white cells in a given volume of blood.

## 7566 P

### Simultaneous Excretion of Creatinine and Certain Organic Compounds of Iodine.\*†

K. A. ELSOM, P. A. BOTT AND E. M. LANDIS (Introduced by A. N. Richards)

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In these experiments the urinary excretion of creatinine has been compared with that of (1) mono-iodo-methane sulphonate of sodium (Skiodan), (2) 3,5-diiodo-4-pyridon-N-acetic acid diethanolamine (Neoskiodan, Diodrast) and (3) sodium ortho-iodohippurate (Hippuran). These 3 organic compounds of iodine owe their practical usefulness in excretion urography to their exceptionally rapid elimination in the urine. Of the normal constituents of urine creatinine is excreted in highest concentration relative to the plasma level. The mechanism of creatinine excretion is not entirely clear (Rehberg,<sup>1</sup> Jolliffe, Shannon and Smith<sup>2</sup>), it is therefore interesting to record that the plasma clearances of these organic compounds of iodine may equal or, under certain conditions, considerably exceed the simultaneously determined creatinine clearances. Their excretion is similar on one hand to that of creatinine, on the other to that of phenol red (Marshall<sup>3</sup>).

In human subjects and unanesthetized dogs various grades of

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\* The expenses of this investigation were defrayed in large part by a grant from the Commonwealth Fund.

† The organic compounds of iodine were supplied by the Winthrop Chemical Company and by the Mallinckrodt Chemical Works.

‡ Determinations of creatinine were made by Miss E. H. Shiels.

<sup>1</sup> Rehberg, P. B., *Biochem. J.*, 1926, **20**, 447, 461.

<sup>2</sup> Jolliffe, M., Shannon, J. A., and Smith, H. W., *Am. J. Physiol.*, 1932, **100**, 301.

<sup>3</sup> Marshall, E. K., Jr., *Am. J. Physiol.*, 1931, **99**, 77.

artefacts, we might well expect both young and old cells to be affected, *i. e.*, cells of all the classes of the polynuclear count. I have made a series of counts to determine (a) whether the non-motile cells occur regularly, and (b) whether they are always old cells, *i. e.*, cells of the higher classes of the polynuclear count. For each count 2 preparations were made under separate coverslips but on the same slide, and these were examined by the supravital technique, counts of non-motile cells being made in regions of the preparations which initially showed many motile forms. Table I shows a typical series of results. The preparation designated by (a) was the one counted first.

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4 a	100	73	2
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5 a	200	75	17
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The nuclei of the non-motile cells became swollen, and the lobation is often obscured. It is possible, in fact, to count the nuclear lobes in only about half the cases. The data on this point are, in consequence, purely qualitative, but where it was possible to distinguish the number of nuclear lobes it was found that there were generally 2 lobes, sometimes one or 3 lobes, and only once 4 lobes. Taken together, the results show that the percentage of non-motile cells show large fluctuations even under the same conditions (*i. e.*, there are large differences between the percentage of these cells in 2 preparations made at the same time from the same sample of blood), whereas the percentage of total polymorphs remains very constant, and also that the non-motile cells are not necessarily old cells. It is a little surprising that a larger number of polymorphs of class IV were not seen, but these, the oldest cells of all, may well have been represented among the non-motiles whose nuclear lobation was impossible to determine. It is to be concluded that the non-motile cells are produced by some uncontrollable factor involved in making the preparation, and that the presence of these cells can not be interpreted as indicating the presence of dying polymorphs in the blood stream.

From purely numerical considerations, it is difficult to see how

simultaneous creatinine and organic iodine clearances, the latter for convenience are expressed in terms of iodine alone. Points on the horizontal line, intercepting the ordinate at 1.0, indicate that the clearances of organic iodine and creatinine were equal. The distance by which a given point is above or below that line shows the amount by which the iodine clearance exceeded or fell short of the simultaneous creatinine clearance, *i. e.*, the magnitude of the ratio,  $\frac{\text{cc. plasma cleared of iodine per minute}}{\text{cc. plasma cleared of creatinine per minute}}$ . This ratio is charted against the average concentration of iodine in plasma during the clearance period.

Skiodan clearances were approximately equal to creatinine clearances over a wide range of plasma Skiodan concentration. Neoskiodan and Hippuran clearances, however, were of the same order of magnitude as the creatinine clearances only when the concentration of Neoskiodan, or Hippuran, in plasma was high. They became several times greater than creatinine clearances when the concentration of Neoskiodan or Hippuran in plasma approached zero.

It appears, therefore, that the mammalian (dog and man) kidney excretes Skiodan and creatinine at approximately the same rate relative to plasma level. Under certain conditions Neoskiodan and Hippuran clearances have the same order of magnitude as the simultaneous creatinine clearances. The mammalian kidney can, however, concentrate Neoskiodan and Hippuran more highly than creatinine when their respective concentrations in plasma approach zero. This relationship provides a new tool for investigating renal function. The mechanism by which the kidney excretes these substances is being studied further.

## 7567 C

### Effects of Deuterium Oxide on Respiration of Germinating Seeds

GEORGETTE J MELOT\* (Introduced by J Ewing)

*From the Biophysical Laboratory, Memorial Hospital, New York City*

Lewis,<sup>1</sup> the first to report on the biological effects of heavy water, states that tobacco seeds do not germinate in nearly pure deuterium oxide, and that they do so very slowly in water containing 50%

\* Fellow of the O R B Educational Foundation.

<sup>1</sup> Lewis, G N, *J Am. Chem. Soc.*, 1933, 55, 3503

water diuresis were induced. Creatinine was administered intraperitoneally (10 to 15 gm) in dogs and orally (30 to 50 gm) in man. Skiodan and Neoskiodan were injected intravenously. Hippuran was administered intravenously in dogs, orally in man. Large amounts of organic iodine were given to dogs but in man the amounts were limited to the ordinary dose used for excretion urography. Urine and samples of blood were collected at half-hourly or hourly intervals.

Urine and separated plasma were analyzed for iodine by Leipert's<sup>4</sup> method, and for creatinine by the method of Folin as used by Holten and Rehberg.<sup>5</sup> The clearances (C) of both iodine and creatinine were calculated in terms of cc of plasma cleared per minute by the usual equation, urinary concentration (U) divided by blood concentration (B) times the volume (in cc) of urine formed per minute (V),  $C = \frac{U \times V}{B}$ .

The excretion of creatinine and organic iodine was independent of the rate of urine formation. Fig 1 shows the relation between

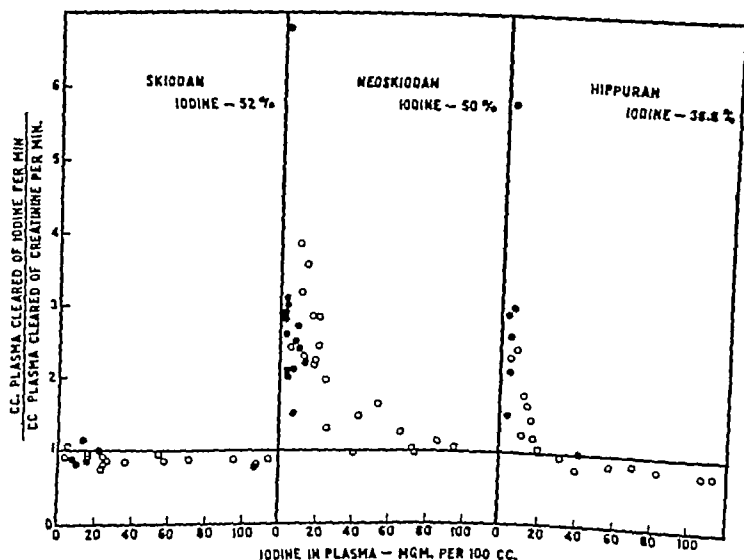


FIG 1

Chart showing ratios between plasma clearances of creatinine and of skiodan, neoskiodan and hippuran, in relation to the concentration of each organic compound of iodine in plasma (expressed in terms of iodine). Dots refer to man, circles to the dog.

<sup>4</sup> Leipert, T, *Biochem Z*, 1933, **261**, 436

<sup>5</sup> Holten, O, and Rehberg, P B, *Acta Med Scand*, 1931, **74**, 479

support satisfactory germination in a given number of seeds. From the figures given below it is evident that 0.1 cc of ordinary water will cause germination in 20 embryos, but that the rate of respiration is significantly higher with 0.15 cc than with 0.1 cc.

As it was difficult to know with accuracy the pH of each sample of heavy water used (since the behavior of the deuterium ion towards indicators is not yet well known), it became necessary to test how far the respiratory rate would depend on changes in the pH due to an impurity as traces of NaOH.

TABLE II  
Respiration of 20 Embryos in Solutions of Different pH

Wt dry embryos	Age	Period of Observation	Vol O <sub>2</sub> consumed	Vol O <sub>2</sub> consumed
			Seeds in 0.1 cc ordinary distilled water pH 5.5	Seeds in 0.0003 N NaOH pH 9.0
mg	hr	min		
78	21½	15	19.7	20.4
		40	55.8	56.5
	34	15	37.3	41.5
		30	76.4	87.7
80	18½	15	22.8	23.4
		30	66.4	65.8
	43	15	35.1	49.6
		30	78.6	107.2

No differences in the respiratory rates of the seeds in the 2 solutions were observed on the first day, but differences began to be apparent on the second day.

Preliminary work was done with 14.8% deuterium oxide. A series of 20 embryos were allowed to germinate for 13 hours in 0.1 cc of ordinary water, another series of 20 embryos in heavy water, and the respiratory rate measured. At this time imbibition was active, but karyokinesis had not begun. An additional 0.1 cc.

TABLE III  
Respiration of 20 Embryos in 14.8% Deuterium Oxide

Wt dry embryos	Age	Period of Observation	Vol O <sub>2</sub> consumed, mm <sup>3</sup>	Seeds in 0.1 cc
			Seeds in 0.1 cc 14.8 % deut. ox.	ordinary distilled water
mg	hr	min		
120	13	15	34.3	36.5
		30	66.5	67.8
		45	97.6	101.4
		60	128.2	136.6
	14	0.1 cc 14.8 deut. ox. and ordinary distilled water added at this time		
		15	35.3	35.2
	40	30	74.6	74.6
		45	113.0	112.0
		60	157.6	156.7

deuterium oxide In view of the new field of research which this finding would open up, if confirmed, it seemed desirable to obtain a quantitative measurement of the effect of deuterium on germination For this reason we have made a study of the rate of aerobic respiration of wheat seeds in the early stages of germination

It is necessary to determine first, whether deuterium oxide inhibits the germination of seeds If so, does it act as a toxic agent, or if mixed with ordinary water, does it simply dilute the ordinary water until the latter is present in insufficient quantity to support germination? If the deuterium oxide acts as a toxic agent, is its action evident before or after the beginning of karyokinesis?

The respiration of wheat seeds under different conditions was followed by means of a Warburg respirometer To bring about germination with very small volumes of water, most of the endosperm portion of the seed, whose rate of respiration is low, was discarded, and the experiments were performed with the end of the seed containing the embryo and a small portion of reserve material, it having been found by experiment that this procedure did not interfere with germination in ordinary water

For each experiment the embryo ends of 10 seeds were placed in the central portion of each of 4 small respirometer (Warburg) vessels Five hundredths cc of heavy water were then added to the seeds in each of the 2 experimental vessels and 0.05 cc of ordinary distilled water to the seeds in each of the control vessels The seeds were allowed to germinate for different lengths of time at 26°C Two-tenths cc of 7% NaOH were then placed in the outer portion of each vessel and the rate of respiration at 26°C was determined over periods The conditions of light and O<sub>2</sub> supply were the same for experimental and control series The experimental error of the method was such that differences between experimental and control figures of less than 10% can not be considered significant

Since the quantity of heavy water available was small, it was necessary to determine the minimum volume of water necessary to

TABLE I.

Respiration of 20 Embryos in Different Quantities of Water 21 Hours after Beginning of Germination

Wt dry embryos mg	Period of observation, Min.	Vol. O <sub>2</sub> consumed, mm. <sup>3</sup> Seeds in 0.1 cc. H <sub>2</sub> O	Seeds in 0.15 cc
102	15	31.5	38.5
	30	64.8	79.4
80	15	31.5	35.0
	30	59.7	68.0
	45	96.4	103.6
	60	118.3	131.8



TABLE V  
Respiration of 20 Embryos in 94% Deuterium Oxide

Wt. of dry embryos	Hr ger mination	Time of ob- servation	mm <sup>3</sup> O <sub>2</sub> consumed					
			Exp 1		Exp 2		Exp 3	
			94% A	Dist B	94% A	Dist. B	94% A	Dist B
mg		hr						
80	0*	1st 1/2	6.9	11.0	2.1	4.1	5.2	8.2
		2nd 1/2	10.0	8.1	4.3	8.2	7.1	7.8
		2nd	18.5	17.9	17.4	20.5	17.6	20.9
		3rd	22.3	25.6	21.5	22.1	23.0	24.7
		4th	26.0	26.8	24.1	26.7	24.2	26.9
		5th			27.5	26.5	27.0	27.2
		6th					30.6	31.0
	24†	1st 1/4	29.4	29.8	{ ----	{ ----	31.9	35.9
		2nd 1/4	29.2	29.8	{ 57.5	{ 59.2	29.8	27.5
		3rd 1/4	34.6	31.5	{ ----	{ ----	{ ----	{ ----
		4th 1/4	30.4	28.3	{ 59.0	{ 57.5	{ 56.4	{ 54.8
	25*	1st 1/4	30.3	36.2	{ ----	{ ----	{ ----	{ ----
		2nd 1/4	30.7	38.5	{ 67.7	{ 80.3	{ 70.8	{ 75.2
		2nd 1/2			74.7	88.9	79.6	92.0
		3rd 1/2			78.3	91.8	111.8	139.2
		4th 1/2			83.6	97.1	164.6	185.2
		5th 1/2			91.5	106.7		
	48	1st 1/4	39.4	40.6			23.5	24.1
		2nd 1/4	38.4	38.2			22.9	24.8
		3rd 1/4	39.1	42.3			25.5	28.4
		4th 1/4	42.5	44.3			23.8	27.3

\*0.1 cc of 94% heavy water and of ordinary distilled water added to the organisms in the experimental (A) and control (B) vessels, respectively. Measurements taken as soon as the apparatus was again in temperature equilibrium.

†Measurements made before any new addition of water.

20 embryos in ordinary water is sensitive to changes in the volume of water available in the range 0.1-0.15 cc, it appears that the heavy water does not act simply as a diluent to ordinary water, but that it enters into the life processes in a manner similar to ordinary water.

**Summary** A study has been made of the germination of wheat seeds (*Triticum vulgare*) in 14.8%, 38% and 94% deuterium oxide, and ordinary water for control under as nearly identical conditions as possible. In all cases the seeds germinated and no macroscopical differences could be detected. No significant differences were observed in the respiratory rates of the seeds in ordinary and heavy water during the first and second days of germination.

The work described in this paper was made possible by the kind co-operation of Dr H. C. Urey who provided the different samples of heavy water.

The author wishes to express to Dr G. Failla and to the members of the Biophysics and Chemistry Departments of Memorial Hospital her appreciation of their assistance during the course of this work.

of water was then placed on the seeds, they were allowed to germinate for another 24 hours, and the respiratory rate was measured again. At this time karyokinesis and visible growth had begun. The results are given in Table III.

No significant difference was observed in the respiratory rates of the seeds in ordinary and heavy water. It is evident from this experiment that deuterium oxide has no pronounced toxic action on the germination of wheat seeds, before or after karyokinesis sets in. The effect of 38% deuterium oxide was then studied.

TABLE IV  
Respiration of 20 Embryos in 38% Deuterium Oxide

Wt dry embryos	Age	Period of Observation	Vol O <sub>2</sub> consumed mm. <sup>3</sup> Seeds in 0.1 cc 38% deut. oxide	Vol O <sub>2</sub> consumed mm. <sup>3</sup> Seeds in 0.1 cc ordinary distilled water
mg	hr	min.		
82	18 30	15	29.0	28.6
		30	55.8	59.3
		45	93.5	94.1
		60	120.9	121.8

Table V shows the results of experiments with 94% deuterium oxide. This material had the same pH as ordinary distilled water when measured under the same conditions.

The question arises whether sufficient ordinary water might have distilled from the NaOH solution into the 94% deuterium oxide to cause germination in the seeds. This is unlikely since the NaOH remained in the Warburg vessels only during the time when respiration measurements were being made, and since the volume (0.2 cc) of NaOH solution used was small. In order to show more definitely that the germination of the seeds in 94% deuterium oxide was not due to such absorption, dry embryos were placed in the inner portion of the Warburg vessel and 0.5 cc NaOH in the outer. The seeds did not take up sufficient water to germinate although observed for several days. Furthermore, it was found that seeds germinated readily in 94% deuterium oxide in a closed vessel containing no NaOH solution.

It is evident that, except for the reading made during the first hour of germination when the percentage experimental error is high, there is no significant difference in the respiratory rates of embryos in ordinary and heavy water during the first 2 days of observation. However, on the second day karyokinesis was active. Since 0.1 cc of 94% heavy water contains only 0.06 cc of ordinary water and since we have shown above that the respiratory rate of

very great, yet the consistency of the biological results from different dilutions bespeaks a fair approximation of the actual to the theoretical cell content

Survivors of small doses were found to be resistant to progressively larger doses until the standard dose (in the order of 80 million cells) was given without harm. In some cases considerable growth of the cells inoculated in the first and second treatment was indicated by large spleens and general sickness followed by recovery, but in most cases there was no clinical evidence of any growth of the inoculated cells.

The results of inoculation with various dilutions and repeated reinoculation of survivors are given in Table I. Numbers in the

TABLE I

Dilute Doses of Leukemic Cells (Line I) in Mice of Strain C58

S = Standard dose, dilutions of standard dose indicated by negative exponents of 4, 2, 3 weeks between successive doses

Standard dose not given			Standard dose given		
Treatment	Death	Survival	Treatment	Death	Survival
—1	4	0	S (3¼ years)	2925	0
—2	4	0	—5, S	0	8
—2 5	4	0	—7, S	2	13
—3	25	0	—7, —3, S	0	1
—4	24	0	—7, —5, S	0	2
—5	24	8	—9, —5, S	1	4
—6	25	7	—6, —5, —3, S	0	1
—7	25	37	—7, —5, —3, S	0	12
—8	2	2	—9, —7, —3, S	0	6
—9	9	83	—9, —7, —5, S	1	9
—9 5	0	48	—10, —7, —5, S	0	4
—10	0	8	—6, —7, —5, —3, S	0	2
—11	0	4	—7, —7, —5, —3, S	0	2
			—7, —9, —7, —5, S	0	1
—9, —5	3	5	—8, —9, —7, —5, S	0	2
—9, —7	3	46	—9, —7, —5, —3, S	0	16
—9, —9	2	24	—9, —9, —7, —5, S	0	2
—10, —7	1	7	—10, —7, —5, —3, S	0	3
			—11, —7, —5, —3, S	0	4
			—9, —9, —7, —5, —3, S	0	22
			Total	114	

columns headed *treatment* indicate dilutions of the standard dose (S) these numbers are negative exponents of 4, so that —1 indicates a dilution of ¼th and —11, 1/4,194,304th of the standard dose. The interval between successive inoculations was usually 2-3 weeks. Mice surviving doses less than standard (entered in the left hand side of the table) in most cases reappear in the right hand side. Animals listed as survivors of the standard dose have shown no sign of leukemia for at least 16 days, this is 4 times the interval

## 7568 C

## Immunization of Mice Naturally Susceptible to a Transplantable Leukemia.

E C MACDOWELL, M J TAYLOR AND J S POTTER

*From the Department of Genetics, Carnegie Institution of Washington, Cold Spring Harbor, New York*

The transplantable leukemia designated as line I<sup>1</sup> was started from a spontaneous case of lymphatic leukemia in April, 1929. The line has passed through 441 transfer generations and, by routine technique, has been inoculated in massive doses into 3625 mice of the highly inbred strain C 58, all but one of these died with the leukemic indications characteristic of this particular line of cells. The single survivor, which at no time showed clinical effects of the inoculation, was inoculated in the 77th transfer generation, in December, 1930. In the 3 $\frac{3}{4}$  years since that time, the 2925 mice from strain C 58 that have been inoculated with the massive standard dose of cells of line I have all developed leukemia. In the light of this record the natural susceptibility of strain C 58 to leukemic cells of line I appears to be fully established. The present experiments, started in April, 1934, are based on 100% susceptibility to the standard dose.

It has been shown previously<sup>2</sup> that between certain limits, reduction of the dosage lengthens the interval before death, and in the early transfers of line I that were used for these experiments (transfers 27-34, Jan-Mar, 1930) the 18 mice given doses of 6000-9000 cells did not die with leukemia. However, these mice were not tested for an immunizing effect of surviving the small doses.

In returning to the study of small doses of the leukemic cells of line I, 346 transfer generations later, the virulence of the cells had become considerably enhanced and it now is found that the minimum dose that will kill is reduced to the order of magnitude of 200 cells. In successive dilutions of the massive standard dose the interval before death is progressively lengthened, as previously reported,<sup>2</sup> but a dilution is reached (1/1024th of standard) that permits a few of the mice to survive and as the dose is further reduced the proportion of survivors increases until every mouse survives (1/524,000th of standard). The percentage variation in cell number of different doses of a highly dilute suspension is probably

<sup>1</sup> Richter, M. N., and MacDowell, E. C., *J. Exp. Med.*, 1930, **52**, 823. Potter, J. S., and Richter, M. N., *Proc. Nat. Acad. Sc.*, 1932, **18**, 298.

<sup>2</sup> Richter, M. N., and MacDowell, E. C., *J. Exp. Med.*, 1933, **57**, 1.

very great, yet the consistency of the biological results from different dilutions bespeaks a fair approximation of the actual to the theoretical cell content.

Survivors of small doses were found to be resistant to progressively larger doses until the standard dose (in the order of 80 million cells) was given without harm. In some cases considerable growth of the cells inoculated in the first and second treatment was indicated by large spleens and general sickness followed by recovery, but in most cases there was no clinical evidence of any growth of the inoculated cells.

The results of inoculation with various dilutions and repeated reinoculation of survivors are given in Table I. Numbers in the

TABLE I  
Dilute Doses of Leukemic Cells (Lane I) in Mice of Strain C58  
S = Standard dose, dilutions of standard dose indicated by negative exponents of 4, 2-3 weeks between successive doses

Standard dose not given			Standard dose given		
Treatment	Death	Survival	Treatment	Death	Survival
—1	4	0	S (3¾ years)	2925	0
—2	4	0	—5, S	0	8
—2 5	4	0	—7, S	2	13
—3	25	0	—7, —3, S	0	1
—4	24	0	—7, —5, S	0	2
—5	24	8	—9, —5, S	1	4
—6	25	7	—6, —5, —3, S	0	1
—7	25	37	—7, —5, —3, S	0	12
—8	2	2	—9, —7, —3, S	0	6
—9	9	83	—9, —7, —5, S	1	9
—9 5	0	48	—10 —7, —5, S	0	4
—10	0	8	—6, —7, —5, —3, S	0	2
—11	0	4	—7, —7, —5, —3, S	0	2
			—7, —9, —7, —5, S	0	1
—9, —5	3	5	—8, —9, —7, —5, S	0	2
—9, —7	3	46	—9, —7, —5, —3, S	0	16
—9, —9	2	24	—9, —9, —7, —5, S	0	2
—10, —7	1	7	—10, —7, —5, —3, S	0	3
			—11, —7, —5, —3, S	0	4
			—9, —9, —7, —5, —3, S	0	22
			Total	114	

columns headed *treatment* indicate dilutions of the standard dose (S), these numbers are negative exponents of 4, so that —1 indicates a dilution of ¼th and —11, 1/4, 194, 304th of the standard dose. The interval between successive inoculations was usually 2-3 weeks. Mice surviving doses less than standard (entered in the left hand side of the table) in most cases reappear in the right hand side. Animals listed as survivors of the standard dose have shown no sign of leukemia for at least 16 days, this is 4 times the interval

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<sup>2</sup> Richter, M. N., and MacDowell, E. C., *J. Exp. Med.*, 1933, **57**, 1.

very great, yet the consistency of the biological results from different dilutions bespeaks a fair approximation of the actual to the theoretical cell content.

Survivors of small doses were found to be resistant to progressively larger doses until the standard dose (in the order of 80 million cells) was given without harm. In some cases considerable growth of the cells inoculated in the first and second treatment was indicated by large spleens and general sickness followed by recovery, but in most cases there was no clinical evidence of any growth of the inoculated cells.

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TABLE I

Dilute Doses of Leukemic Cells (Line I) in Mice of Strain C58

S = Standard dose, dilutions of standard dose indicated by negative exponents of 4, 2-3 weeks between successive doses

Standard dose not given			Standard dose given		
Treatment	Death	Survival	Treatment	Death	Survival
—1	4	0	S (3¾ years)	2925	0
—2	4	0	—5, S	0	8
—2 5	4	0	—7, S	2	13
—3	25	0	—7,—3, S	0	1
—4	24	0	—7,—5, S	0	2
—5	24	8	—9,—5, S	1	4
—6	25	7	—6,—5,—3, S	0	1
—7	25	37	—7,—5,—3, S	0	12
—8	2	2	—9,—7,—3, S	0	6
—9	9	83	—9,—7,—5, S	1	9
—9 5	0	48	—10,—7,—5, S	0	4
—10	0	8	—6,—7,—5,—3, S	0	2
—11	0	4	—7,—7,—5,—3, S	0	2
			—7,—9,—7,—5, S	0	1
—9,—5	3	5	—8,—9,—7,—5, S	0	2
—9,—7	3	46	—9,—7,—5,—3, S	0	16
—9,—9	2	24	—9,—9,—7,—5, S	0	2
—10,—7	1	7	—10,—7,—5,—3, S	0	3
			—11,—7,—5,—3, S	0	4
			—9,—9,—7,—5,—3, S	0	22
			Total 114		

columns headed *treatment* indicate dilutions of the standard dose (S), these numbers are negative exponents of 4, so that —1 indicates a dilution of ¼th and —11, 1/4,194,304th of the standard dose. The interval between successive inoculations was usually 2-3 weeks. Mice surviving doses less than standard (entered in the left hand side of the table) in most cases reappear in the right hand side. Animals listed as survivors of the standard dose have shown no sign of leukemia for at least 16 days, this is 4 times the interval

between inoculation and death of the controls. At present 58 survivors of the standard dose have been under observation for at least 3 months.

Although many variations in procedure prevent any simple statement of the degree of success obtained, the phenomenon of active immunization of mice naturally susceptible to massive doses of a certain line of leukemic cells appears to be established by the 114 mice that have been immunized to resist the standard dose, in contrast to the 2925 non-immunized mice of the same strain that have been inoculated since 1930 with the same standard dose without a single survivor.

## 7569 C

## Vaccination of Rabbits Against Intradermal Pneumococcus Infection

JOHN A. KOLMER AND ANNA M. RULE

*From the Research Institute of Cutaneous Medicine of Philadelphia*

A vaccine of Type I pneumococcus was prepared by cultivating a highly virulent strain in broth for 24 hours at 37°C. The culture was thoroughly centrifuged, the supernatant broth discarded and the pneumococci suspended in sterile distilled water to give approximately 1000 million per cc. This suspension was heated at 60°C for one hour, cultured for sterility and preserved with 0.3% tricresol.

Similar vaccines were prepared of Type II and Type III pneumococci.

*Results with Type I Vaccine.* Six adult rabbits were given 1 cc of Type I pneumococcus vaccine per kilo of weight by subcutaneous injection every 5 days for 5 doses. One week after the last dose all, along with 2 controls, were inoculated intradermally with 0.2 cc of 18-hour broth culture after the method of Goodner.<sup>1</sup> The controls developed the typical local lesions, associated septicemia, fever, leukocytic changes, etc., and succumbed 4 to 5 days after the inoculation.

All of the 6 vaccinated rabbits survived. Local lesions of slight to moderate severity developed in all with positive cultures of the edema fluid over 1 to 2 days following inoculation which were thereafter sterile. Daily blood cultures were negative in 5 animals,

<sup>1</sup> Goodner, K., *J. Exp. Med.*, 1928, **48**, 1, 413, 1931, **54**, 847.



in 1 the blood culture was positive 24 hours after inoculation and thereafter negative. Mild to moderate fever with slight leukocytosis developed in all over a period of about 3 days and then reached normal.

Two rabbits were given 2 cc. of the vaccine per kilo every 5 days for 5 doses by subcutaneous injection. When inoculated intradermally one week after the last dose both survived with negative blood cultures throughout. The local lesions were mild with positive cultures of the edema fluid only once 24 hours after inoculation in both animals.

Four rabbits were given 1 cc. of the vaccine per kilo every 5 days for 5 doses by stomach tube and inoculated intradermally 5 days after the last dose. Three developed typical local lesions with the associated septicemia, fever, leukocytic changes, etc. and died about 4 days after inoculation. One developed a local lesion of moderate severity with 2 positive blood cultures but survived.

Four additional animals received 2 cc. per kilo every 5 days for 5 doses by stomach tube. When inoculated intradermally 5 days after the last dose 2 survived after moderate local lesions with 2 positive blood cultures, while the remaining 2 died with severe local lesions, septicemia, etc. about 5 days after inoculation.

Mouse serum protection tests were not conducted because as stated by Goodner and Stillman<sup>2</sup> the 2 factors do not necessarily parallel each other.

*Results with Type II Vaccine* Two rabbits were given 1 cc. and 2 additional animals 2 cc. of vaccine per kilo every 5 days for 5 doses by subcutaneous injection. Two were given 1 cc. and 2 additional animals 2 cc. per kilo every 5 days for 5 doses by stomach tube. Five days after the last dose all, including 2 controls, were given an intradermal injection of 0.5 cc. of an 18-hour broth culture in 2 injections closely spaced.

Both controls and all vaccinated animals survived. Among the 4 animals receiving subcutaneous injections of the vaccine the local lesions, however, were much less severe. Among the 4 receiving the vaccine by stomach tube the local lesions were approximately equal to the controls.

All animals including the controls gave 1 or 2 positive blood cultures during 72 hours following inoculation but thereafter sterile cultures. Among the vaccinated animals and particularly those receiving subcutaneous injections of vaccine, the fever and leukocytosis were appreciably less than observed with the controls.

<sup>2</sup> Goodner, K., and Stillman, E. G., *J. Exp. Med.*, 1933, 58, 195.

*Results with Type III Vaccine* Two rabbits were given 1 cc and 2 additional animals 2 cc of vaccine per kilo every 5 days for 5 doses by subcutaneous injection. Two were given 1 cc and 2 additional animals 2 cc per kilo every 5 days for 5 doses by stomach tube. All, including 2 controls, were given an intradermal inoculation of 0.5 cc of an 18-hour broth culture 5 days after the last dose.

All of the vaccinated animals and one control survived while the remaining control succumbed with a severe local lesion, septicemia and associated fever and leukocytic changes. The surviving control developed a moderately severe local lesion with 2 positive blood cultures. The 2 animals receiving subcutaneous injections of 1 cc of vaccine developed moderately severe local lesions with 2 positive blood cultures but the 2 receiving the 2 cc dose developed milder local lesions with sterile blood cultures. The 2 animals receiving the 1 cc dose of vaccine per kilo by stomach tube developed moderate local lesions and positive blood cultures (1 had 2 and the second 4) but ultimately recovered. The 2 receiving the 2 cc dose per kilo also had moderate local lesions with 2 positive blood cultures but also survived.

Goodner<sup>1</sup> has reported that one intravenous injection of 10 cc of an extremely heavy suspension of washed heat-killed vaccine of Type I pneumococcus vaccine protected rabbits against the local and associated lesions and found protective antibody in the blood 5 days later. In only 1 rabbit out of 8 was this immunity found to last for 2 months. Stillman and Goodner<sup>2</sup> have also given 6 rabbits intravenous injections of a heat-killed vaccine containing Type I, II and III in equal proportions. All survived when inoculated intradermally after the last injection, whereas 3 controls died 1 to 4 days after inoculation. Additional rabbits given intravenous injections of pneumococcus autolysate developed a lesser degree of resistance to intradermal infection. The active immunity was found highly specific for the homologous pneumococci and very low to heterologous types.

We preferred to use subcutaneous injections because adapted to practical use in the immunization of human beings. Oral immunization was also employed because previous investigations by Ross<sup>3</sup> have shown this route is effective in rats and we have also found it somewhat effective in the immunization of rabbits<sup>4</sup> and monkeys.<sup>5</sup>

<sup>1</sup> Ross, V. J., *J. Exp. Med.*, 1930, **51**, 585, *J. Immunol.*, 1926 **12**, 219, 237.  
<sup>2</sup> Stillman and Goodner, *J. Lab. and Clin. Med.*, 1927, **12**, 566, *Proc. Soc. Exp. Biol. and Med.* 1926, **24**, 273.

<sup>3</sup> Kolmer, J. A., and Rule, A. M., *Proc. Soc. Exp. Biol. and Med.* 1932 **30**, 107, 1933, **31**, 245.

Apparently very large doses of vaccine are required by either route of administration and the resulting immunity is apparently of short duration.

*Summary* 1 Rabbits have been successfully vaccinated by 5 subcutaneous injections of a heat-killed aqueous suspension of Type I pneumococcus vaccine against fatal intradermal pneumococcus infection 2 When the vaccine was given by stomach tube about 38% of rabbits survived 3 With Type II vaccine all animals including the controls survived following intradermal pneumococcus infection but the local lesions, septicemia and associated febrile and leukocytic changes were less marked among the immunized animals and especially those receiving 5 subcutaneous injections of vaccine 4 All rabbits immunized with Type III vaccine by subcutaneous and oral administration survived along with 1 out of 2 controls following intradermal infection The local lesions and associated fever and leukocytosis were milder among the vaccinated animals than in the controls and especially among those immunized with subcutaneous injections of vaccine

### 7570 C

#### Chemotherapy of Intradermal Pneumococcus Infection of Rabbits. Effects of Optochin and Other Quinine Compounds

JOHN A. KOLMER AND ANNA M. RULE

*From the Research Institute of Cutaneous Medicine of Philadelphia*

Rabbits were inoculated intradermally with 0.2 cc of 18-hour broth cultures of a highly virulent Type I pneumococcus after the method of Goodner.<sup>1</sup>

Immediately after 2 rabbits were given 0.005 gm *optochin base* (Merck) and 2 additional animals 0.01 gm per kilo by stomach tube and the doses repeated every 6 hours over a period of 3 days. There was no appreciable influence upon the local lesions, temperatures, leukocytic changes, positive blood cultures or cultures of edema fluid. All animals succumbed in from 4 to 5 days.

Two rabbits were given *optochin base* in dose of 0.01 gm per kilo every 6 hours for 4 doses before intradermal inoculation with pneu-

<sup>1</sup> Kolmer, J. A., and Rule, A. M., *Proc. Soc. Exp. Biol. and Med.* 1933, **31**, 243.

<sup>2</sup> Goodner, E., *J. Exp. Med.*, 1928, **48**, 1, 413, 1931, **54**, 517.

mococcus and then every 6 hours thereafter for 4 additional doses. The compound was without appreciable effect upon the local lesions, associated septicemia, etc., and both succumbed in 4 days.

Two rabbits were given *optochin base* suspended in oil by intramuscular injection in dose of 0.01 gm per kilo immediately after intradermal inoculation with pneumococcus and repeated every 24 hours for 3 additional doses. Both succumbed about 4 days later with no appreciable effects upon the local lesions, associated septicemias, etc.

Two rabbits were given *ethyhydrocuprein hydrochloride* dissolved in water by intramuscular injection in dose of 0.01 gm per kilo immediately after intradermal inoculation with pneumococcus and repeated daily for 2 additional doses. Both animals succumbed between 3 and 4 days after inoculation with no appreciable effects upon the local lesions, septicemias, etc.

Two rabbits were given *quinine* and *urea hydrochloride* by stomach tube in dose of 0.01 gm per kilo immediately after intradermal inoculation with pneumococcus and the dose repeated every 6 hours for 6 additional doses. Two additional animals were given the same compound in the same dosage by intramuscular injection immediately after intradermal inoculation and repeated every 6 hours for 4 additional doses. All 4 animals succumbed in about 4 days with no appreciable effects upon the local lesions and associated septicemias, fever, leukocytosis, etc.

Two untreated controls inoculated intradermally at the same time developed the typical local lesions with daily positive cultures of edema fluids, daily positive blood cultures, leukocytic and febrile changes and succumbed between 4 and 5 days after inoculation.

*Summary* Optochin base, ethyhydrocuprein hydrochloride and quinine and urea hydrochloride administered by stomach tube and by intramuscular injection in repeated doses had no appreciable curative effects upon the local lesions, associated septicemia, fever or leukocytic changes induced in rabbits by the intradermal inoculation (Goodner) of virulent type I pneumococcus.

## 7571 P

## Sulphates of Sodium and Magnesium on Gastro-intestinal Activity

GEORGE B ROTH AND PHOEBE J CRITTENDEN

*From the Department of Pharmacology and Therapeutics, The George Washington University*

Our experiments were designed to ascertain the effects of the sulphates of sodium and magnesium on the gastro-intestinal tract of unanesthetized dogs, each having a gastric and intestinal fistula. Rozen and Perusse<sup>1</sup> using unanesthetized dogs with a gastric fistula reported on gastric activity several hours after the use of magnesium chloride but other similar work has not been noted.

Two female dogs, S and B, were used as subjects. The fistulae were made after the manner of Thiry<sup>2</sup> (intestinal) and Carlson<sup>3</sup> (gastric) in a 2 stage operation. The animals were in good physical condition. Food was withheld for 18 hours prior to an experiment, but water was allowed *ad libitum*. Both subjects were trained to lie quietly during the experiments. Our experiments will be described under 2 series since the technic in each differed.

*Series I*—This series deals with the effects of the sulphates of sodium and magnesium on gastro-intestinal activity when the salines were placed in the stomach, graphic records (smoked paper) being obtained from both stomach and intestinal segment by means of balloons and soap water manometers. The salines were usually given after a normal record of about 1 hour had been procured.

The controls for this series were of 2 kinds (a) with the stomach empty and (b) with the stomach containing 0.85% sodium chloride.

*Results* Isotonic sodium or magnesium sulphate (25 to 50 cc amounts) in contact with the gastric mucosa was without noteworthy effect on gastro-intestinal activity. Hypertonic (5 times) solutions of magnesium sulphate, however, depressed both gastric and intestinal movements slightly but were without effect on intestinal activity. Hypertonic sodium sulphate (5 times) appeared to increase intestinal activity about 30%.

*Series II*—Series II deals with the effects of the above salines on the gastro-intestinal activity of dogs S and B when the salines were

<sup>1</sup> Rozen, J. S., and Perussé, G. L., *Am J Physiol* 1929, **91**, 293.

<sup>2</sup> Thiry, *Sitzgsber d Wiener Acad Math Nat Hist*, 1864, **50**, 77.

<sup>3</sup> Carlson, A. J., "The Control of Hunger in Health and Disease," University of Chicago Press, 1916, 42.

placed in the loop of the intestinal fistula. As before, simultaneous records of the gastric and intestinal movements were taken, the gastric movements being recorded by the balloon method and the intestinal activity by a new closed system method in which the intestinal fistula was directly connected to the manometer. The essential parts in this system were fistula—reservoir—manometer. A small stopper carrying a glass tube was placed in the opening of the fistula (dog B) or a rubber tube over a glass tube (dog S). Between the manometer and the fistula was a reservoir into which the solution from the intestinal fistula could flow when contractions occurred and from which the solution could drain into the segment of intestine when relaxation occurred. The manometer was the same type used in the first series. This method of recording intestinal activity we believe is essential, since there is no escape of fluid as in the balloon method, thus making long observations possible.

The results obtained were from experiments of 4 hours duration in which the sulphate solutions were either interspersed between physiologic saline or following it, each saline being left in the intestinal fistula for a period of about 1 hour. The hypertonic solutions were never more concentrated than twice hypertonic. Distilled water was also used in a few experiments.

*Results Stomach*—no noteworthy effects

*Intestines*—*Hypertonic sodium chloride and distilled water*. Distilled water in general caused only a slight increase in movements, except in 2 experiments in "B" the amplitude was doubled when used following isotonic sodium chloride. Hypertonic sodium chloride increased the amplitude over the normal but slightly in some instances, whereas in others the amplitude was increased 8 times that of the control, the average being about 3 times. There was also a temporary increase for a few minutes in the fluid content of the intestines.

*Isotonic sodium sulphate and isotonic magnesium sulphate*. The isotonic sodium sulphate stimulated the activity to twice that of the control sodium chloride regardless of whether it preceded or followed the magnesium sulphate. The magnesium sulphate stimulated the activity to 4 times that of the control. When isotonic sodium chloride followed isotonic magnesium sulphate the usual basic effects of isotonic sodium chloride were absent. Both isotonic magnesium and sodium sulphate increased the fluid content of the

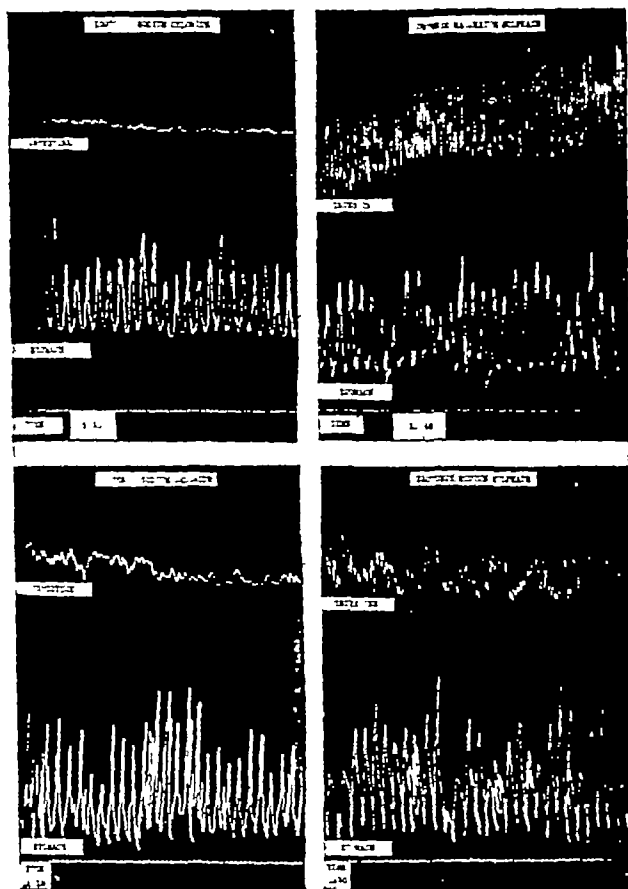


FIG 1

Effects of various isotonic salines on gastric intestinal loop activity on un anesthetized dog (Brindle 12½ kg Female 12/22/33)

Time in 6 seconds Tracings as follows Upper left, isotonic sodium chloride, upper right, isotonic magnesium sulphate, lower left, isotonic sodium chloride, lower right, isotonic sodium sulphate

The upper tracings were taken one half hour, lower tracings about 45 minutes, after the salines were placed in the intestinal loop

intestines, the effects lasting throughout the experiment. The magnesium effect was the more marked

*Hypertonic sodium sulphate and hypertonic magnesium sulphate*  
The magnesium sulphate (twice hypertonic) caused a stimulation of intestinal activity 9 to 10 times that of the control (isotonic sodium chloride) The sodium sulphate (twice hypertonic) increased the activity about 8 times when it preceded the magnesium, and about 12 times when it followed the magnesium

When isotonic sodium chloride followed hypertonic magnesium sulphate the usual contractions present during sodium chloride were absent in some experiments and present in others. The fluid content of the intestine was markedly increased with each of the hypertonic solutions but slightly more so after the magnesium. The magnesium also appeared to lower the tone of the intestine.

Specific effects of both magnesium and sulphate ions have thus been demonstrated as well as the usual osmotic effects.

## 7572 P

## Determination of Lactic Acid in Presence of Certain Interfering Substances.

EVAN W. MCCHESENEY (Introduced by W. deB. MacNider)

*From the Department of Physiology, University of North Carolina*

Probably the best technique now available for the determination of lactic acid is that of Friedemann, Cotonio, and Shaffer<sup>1</sup> as modified by Friedemann and Kendall<sup>2</sup> and, more recently, by Friedemann and Graesser<sup>3</sup>. There are certain substances which interfere with the lactic acid determination, these have been listed in the original and some of the subsequent articles. Some of these substances, notably the sugars, may be removed from solution by the copper sulfate-calcium hydroxide procedure<sup>2</sup>. Appreciable amounts of others, however, remain in solution even after this treatment. Important examples of these substances are malic and citric acids, both of which give large yields of bisulfite-binding substances under the conditions used for the oxidation of lactic acid to acetaldehyde. In fact, by some slight modifications of the conditions it is possible to obtain very nearly a quantitative yield of acetaldehyde from malic acid (90 to 93%)<sup>4</sup>. The product formed by the oxidation of citric acid is apparently acetone<sup>5</sup> and by the lactic acid method a yield of about 60% of the theoretical may be obtained<sup>4</sup>. However, both malic and citric acids may be quite completely removed from solu-

<sup>1</sup> Friedemann, T. E., Cotonio, M., and Shaffer, P. A., *J. Biol. Chem.*, 1927, **73**,

335

<sup>2</sup> Friedemann, T. E., and Kendall, A. I., *J. Biol. Chem.*, 1929, **82**, 23

<sup>3</sup> Friedemann, T. E., and Graesser, J. B., *J. Biol. Chem.*, 1933, **100**, 291

<sup>4</sup> McCchesney, E. W., unpublished observations

<sup>5</sup> Kuyper, A. G., *J. Am. Chem. Soc.*, 1933, **55**, 1722



tion by means of basic lead acetate as follows. An amount of solution thought to contain 20-50 mg of lactic acid and any reasonable amount of the interfering substance is placed in a 100 cc volumetric flask, and diluted to a volume of about 50 cc. A drop of aqueous phenol red is added, then 10% NaOH is added drop by drop until the solution is neutral followed by one drop excess. A saturated solution of basic lead acetate (Haden) is added in 5 cc portions until there is an excess, usually 5 cc is sufficient and a large excess is to be avoided. Zinc ions should be absent from the solution as some precipitation of lactic acid seems to result if they are present. The solution is now diluted to the mark, mixed, and filtered. Lactic acid is determined in the usual way on aliquot portions of the filtrate. With the addition of the  $\text{MnSO}_4\text{-H}_3\text{PO}_4$  reagent, a precipitate forms and this causes some bumping during the subsequent boiling but does not seem to interfere otherwise. Table 1 shows the completeness of the separation of lactic and malic acids by this method.

TABLE I.

Mg lactic acid in 100 cc vol.	Mg malic acid added	Mg lactic acid found
40.8	0	40.8
0	100	0.8
20.4	100	20.3
40.8	100	40.1
81.6	100	78.6
122.4	100	119.4
40.8	20	40.2
40.8	50	40.0
40.8	75	40.2

The average recovery of lactic acid from the mixtures was 98%.

*Other substances.* Of the other substances reported in the original article<sup>1</sup> to interfere with the lactic acid determination, the following have been found to give precipitates with basic lead acetate when 10 mg of the substances are present in the final volume of 100 cc. tartaric acid, citric acid, tyrosine, cystine, and maleic acid. The following have been found to yield precipitates with basic lead acetate when 5 mg of the substance are present in the final volume of 100 cc. tartaric acid, citric acid, tyrosine, and cystine (trace). The sugars glucose, fructose, xylose, galactose, and arabinose are of course not precipitated. This method is not likely to be of value in eliminating the various interfering alpha-hydroxy acids since in addition to lactic acid, alpha-hydroxy isovaleric acid is not precipitated, nor is the acid derived from the deamination of leucine (alpha-hydroxy isocaproic acid).

## Virulence of *Trichinella Spiralis* in a Natural and in an Experimental Host

G W BACHMAN AND J OLIVER. (Introduced by S A. Koser)

From the Department of Parasitology of the School of Tropical Medicine of the University of Puerto Rico, under the auspices of Columbia University

Measurements were made by passing an active strain of *Trichinella spiralis* consecutively through 2 series of white rats and rabbits at regular intervals. In one series (Series A) comprising 14 rats and 10 rabbits, the strain was passed successively from rat to rat and from rabbit to rabbit for a period of 7 months at intervals of 20 days each. The initial dose of trichinous meat was taken from an experimentally infested rabbit, and in each case consisted of a sub-lethal dose, determined previously on another series of animals. At the end of the 20-day period, the rat and rabbit were killed, and a sub-lethal quantity of their trichinous flesh was then fed to a normal rat and rabbit respectively, i. e., the rat flesh to the rat, and the rabbit flesh to the rabbit. This process was repeated until the *Trichinella spiralis* strain had completely died out or could not be detected in the rabbits, which happened after the fifth feeding, but in the white rats, on whom the same technique had been used, the number of worms progressively increased and the worms retained their power to reinfect normal rats after having been passed through rats in succession for nine months.

In the second series (Series B) of 13 rabbits and 22 rats, the same experiments were made as before, except that in this case the initial trichinous meat was taken from a rat instead of from a rabbit. In this series, as in the former experiment, the strain died out in the rabbits at the end of the fifth feeding while again, in the rats the number of worms per gram of meat continued to increase, and retained their power of penetrating the muscles of new hosts after a period of 11 months.

From this experiment, we may conclude that when the worms invade the natural host they adapt themselves to conditions in the living body and retain their virulence and power to penetrate muscles. They seem to lose virulence and numbers on successive passage through the experimental or unnatural host.

## 7574 C

## Clotting of Plasma in the Absence of Lipoid

HSIEN WU

*From the Department of Biochemistry, Peiping Union Medical College*

Cephalin is known to accelerate clotting of blood. In Howell's theory<sup>1</sup> of blood coagulation a thromboplastic substance (or thromboplastin) is liberated from the platelets or tissues when the blood is shed. This thromboplastic substance is supposed to combine with anti-prothrombin (heparin<sup>2</sup>), setting free prothrombin which is converted by calcium into thrombin. The thromboplastic substance is fat-soluble and appears to be cephalin. The theories of Morawitz<sup>3</sup> and of Bordet<sup>4</sup> are essentially similar to that of Howell. The thrombokinas of Morawitz is only a different name for the same substance as the thromboplastin of Howell, while the cytzyme of Bordet is believed to be a lipoprotein. In Mill's theory<sup>5</sup> of blood clotting, thrombin is a protein-calcium-cephalin compound and the fibrin is a protein-calcium-cephalin-fibrinogen compound. If this view is correct, clotting is impossible in the absence of cephalin, while according to the other theories clotting can still occur in the absence of lipid, once the prothrombin has been converted into thrombin.

Hardy and Gardiner<sup>6</sup> found that plasma which had been freed from lipoids by extraction with alcohol in the cold clotted normally. This seems to suggest that lipid is not necessary for clotting. However, this observation of Hardy and Gardiner was only incidental to their study of fat-free serum proteins and they did not mention how the clotting was induced. Their clotting mixture must have contained some lipid because the clotting agent, be it serum or thrombin, prepared in the usual way, could not be lipid-free. It would be desirable to study the clotting phenomenon in the entire absence of lipid.

*Preparation of material.* Lipoid-free plasma and serum were prepared essentially according to Hewitt's method<sup>6</sup> described in detail elsewhere. Oxalated plasma was used. In one experiment the ox-

<sup>1</sup> Howell, W. H., *Bull. Johns Hopkins Hosp.*, 1928, **42**, 199.

<sup>2</sup> Morawitz, P., *Ergeb. Physiol.* 1905, **4**, 307.

<sup>3</sup> Bordet, J., *Ann. L. Inst. Pasteur*, 1920, **34**, 561.

<sup>4</sup> Mills, C. A., and Guest, G. M., *Am. J. Physiol.*, 1921, **57**, 395.

<sup>5</sup> Hardy, W. B., and Gardiner, S., *J. Physiol.*, 1910, **40**, lxxviii.

<sup>6</sup> Hewitt, L. F., *Biochem. J.*, 1927, **21**, 216.

late was removed by dialysis against 0.8% NaCl before the removal of lipid. The result was essentially the same.

For coagulation experiments, the plasma or serum powder was dissolved in 0.8% NaCl solution to give a concentration of 7%. The solutions were centrifuged to remove any insoluble (denatured) protein.

The tests were carried out as follows. 28 cc of 0.8% NaCl were mixed with

- a 1 cc natural serum
- b 1 cc fat-free serum
- c 1 cc 2.5%  $\text{CaCl}_2$
- d 5 drops of 5% ethereal solution of plasma lipid. This was recovered from the ether-alcohol filtrate in the preparation of fat-free plasma and serum. Most of the lipid was precipitated when the ethereal solution was added to the saline. The precipitate was removed by filtration.
- e 1 cc 2.5%  $\text{CaCl}_2$  and 5 drops of 5% ethereal solution of plasma lipid.

To each of the above mixtures 1 cc of fat-free plasma was added. In a similar series of experiment, natural plasma was used. The results are shown in the accompanying table.

TABLE I  
Coagulation of natural plasma and lipid free plasma

Clotting agent	Natural plasma	Fat free plasma
Natural serum	+	
Lipoid free serum	+	+
Calcium	+	+
Lipoid	—	—
Calcium lipoid	+	—
		+

+ Indicates clotting

The clotting of fat-free plasma with natural serum or natural plasma with fat-free serum is to be expected, since in these mixtures all the plasma constituents are present. The clotting of the fat-free plasma with fat-free serum demonstrates that lipid is not essential for the formation of the clot. However the fat-free plasma does not clot on calcification as the natural plasma does. Only when both calcium and lipid are added was the clotting of the fat-free plasma induced. Addition of lipid alone to the plasma could not induce clotting.

*Conclusion* From these observations we must conclude that lipid as well as calcium is essential for the activation of the thrombin but once this is formed the presence of lipid is not necessary.

## 7575 C

Differences in Susceptibility to Ultraviolet Radiation of *Paramecium Caudatum* and *P. Bursaria*.

W W ALPATOV AND O K. NASTJUKOVA.

From the Laboratory of Ecology, Institute of Zoology, University of Moscow

I In our previous paper<sup>1</sup> we were able to show that the influence of ultraviolet radiation on *P. caudatum* can be expressed by the Arndt-Schultze's law. Since the body of *P. bursaria* is full of green symbiotic algae, it seems worth while to compare the susceptibility of 2 species of Infusoria, differing in body coloration and hence in absorption of radiation.

TABLE I.  
Averages and Probable Errors of the Numbers of Offspring of Infusoria in the First Series of Experiments

Duration of radiation sec	<i>Paramecium caudatum</i>	<i>Paramecium bursaria</i>
Control	100.2 $\pm$ 1.77	97.6 $\pm$ 2.00
40	85.6 $\pm$ 1.67	110.4 $\pm$ 3.39
80	69.0 $\pm$ 1.70	98.4 $\pm$ 1.18
160	45.8 $\pm$ 1.47	74.0 $\pm$ 2.61

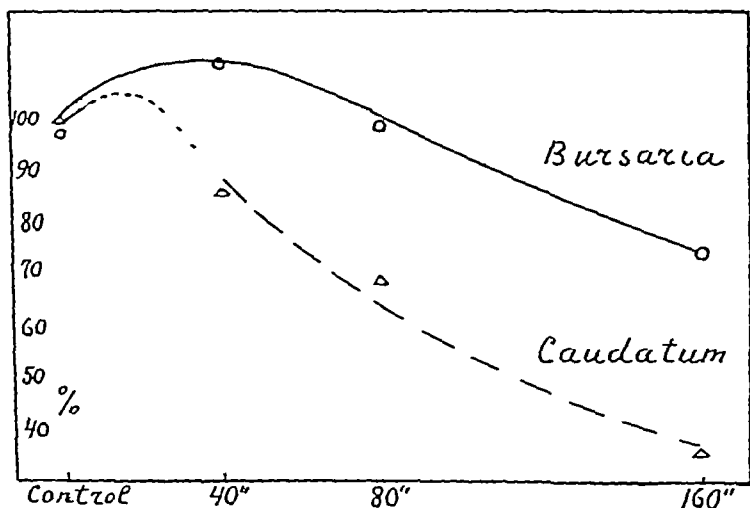


FIG 1

Curves showing the relationship between the division rate and the doses of the ultraviolet radiation. The dotted part of the curve for *P. caudatum* is founded on the data of our preceding paper (1933).

<sup>1</sup> Alpatov, W W, and Nastjukova, O K., *Protoplasma*, 18, No. 2

2 As a source of light was taken a quartz mercury vapour burner of Hereus Hanau, 110 volts of alternating current and 6.7 amp at a distance of 35 cm from the animals under quartz Petri dishes filled with water 0.5 cm deep. The control animals were put under glass dishes to absorb the ultraviolet part of the spectrum. An oatmeal medium containing *Bacillus subtilis* was used. The first series consisted of 5 separate experiments of about 160 specimens each. After keeping them for 24 hours at a temperature of 25°C on slides with hollows their progeny were counted. The second series of experiments consisted in submitting the same cultures to repeated radiations on consecutive days, keeping the number of parental animals equal to 192. The number of the progeny of each specimen in the first series was expressed in per cent of the average number of the progeny of the control of the corresponding experiment.

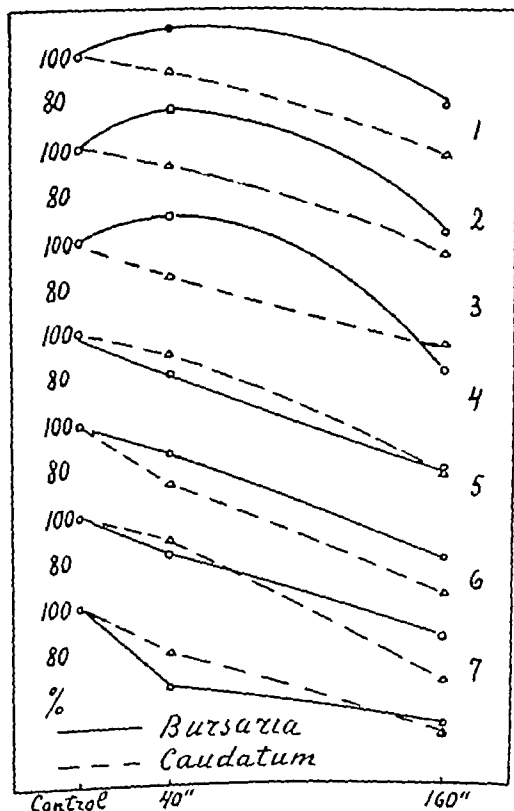


FIG 2

Curves showing the results of radiations repeated on 7 consecutive days

These data show that a radiation of 40 seconds produces in *P caudatum* a depression of the division rate and the zone of stimulative action is therefore located between 0 and 25 seconds. The curve for *P bursaria* goes above that of *P caudatum* and correspondingly the zone of stimulation is extended more to the right somewhere between 0 and 80 seconds. On the whole *P caudatum* is about 2 times more susceptible to the stimulative and depressive action of the ultraviolet radiation as compared with *P bursaria*.

The second series of experiments during the first 3 days confirms these conclusions.

Beginning with the fourth day the susceptibility of *P bursaria* turned out to be practically the same as that of *P caudatum*. The explanation is as follows. During the whole period of experimentation the cultures were kept in darkness and microscopical observation has shown that *P bursaria* has lost almost completely the green color of the symbiotic algae. It seems therefore that the presence of pigmented algae is the cause of a greater resistance of *P bursaria* against the influence of the ultraviolet radiation.

## 7576 C

### Oestrus in Hypophysectomised Rats Parabiotically Connected with Castrates \*

EMIL WITSCHI AND W. T. LEVINE

*From the State University of Iowa*

It is known that unoperated female rats, when united in parabiosis with castrates, first pass through a period of irregular oestral and anoestral activity and later go into a condition of constant oestrus. Hill<sup>1</sup> has reported that during this second period the ovaries always contain large numbers of mature follicles but no corpora lutea. On the contrary in the first period the ovaries are crowded with corpora lutea which, especially during prolonged anoestral phases, are increased in number far beyond anything observed under normal conditions. Cryptorchid males (Martins<sup>2</sup>) as

\* This investigation was supported by grants from the Committee for Research in Problems of Sex of the National Research Council.

<sup>1</sup> Hill, R. T., *Endocrinol.*, 1933, **17**, 414.

<sup>2</sup> Martins, Th., *Compt. Rend. Soc. de Biol.*, 1930, **105**, 789.

well as X-ray sterilized males and females (Levine and Witschi<sup>3</sup>) produce the same effects as castrates (Fig 6) if put in parabiosis with normal females. This is remarkable since the secondary sex characters are maintained in these cases. An histological examination of the hypophyses proves that the characteristic "castrate cells" are present not only in castrates but also in cryptorchids (Fig 7) and in X-rayed males and females (Levine and Witschi<sup>3</sup>). Destruction of the germ cells by either treatment causes similar or identical histological and functional changes in the hypophysis.

Fels<sup>4</sup>, Kallas<sup>5</sup>, and Martins<sup>6</sup> suggested that the known hyper-function of the "castrate hypophysis" might be responsible for the striking reactions in the unoperated parabiont. These authors gave little consideration to the fact that the non-castrate too possesses a hypophysis which may participate in evoking ovarian reactions. The experiments here described will furnish proof that each hypophysis is responsible for one of the 2 main reactions, prolonged anoestrus and constant oestrus.

In a first experiment it was attempted to unite females hypophysectomised for over one month with male castrates. This was not very successful. The females, which were weakened by hypophyseal deprivation, died in all but one case shortly after the operation. In the one surviving case the female recovered to a healthy condition only after several weeks. She remained in anoestrus during the first month of parabiosis and then went into constant oestrus. Obviously, the anoestral phase was due to the poor general condition of the female, following the parabiosis operation. The oestral phase, which has lasted now for 2 months with only one short interruption, must be due to the influx of hypophyseal hormones from the castrate.

A second experiment starts with females that had been in parabiosis with castrate males from 2 to 6 months. All had established the constant oestrus condition. In 5 pairs of this type the hypophysis of the female was removed with no effect upon the oestral type of vaginal smears (Fig 1, pair 192). It is true that some of the females fell once or twice into anoestrus for periods of 1 to 3 days, immediately following hypophysectomy, though apparently this was due merely to general disturbances caused by the operation.

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<sup>3</sup> Levine, W T, and Witschi, E, *Proc Soc Exp Biol and Med*, 1933, **30**, 1152

<sup>4</sup> Fels, E, *Arch f Gynaek*, 1929, **138**, 16

<sup>5</sup> Kallas, H, *Pflüg Arch ges Physiol*, 1929, **223**, 232

<sup>6</sup> Martins, Th, *Compt Rend Soc de Biol*, 1929, **103**, 1341



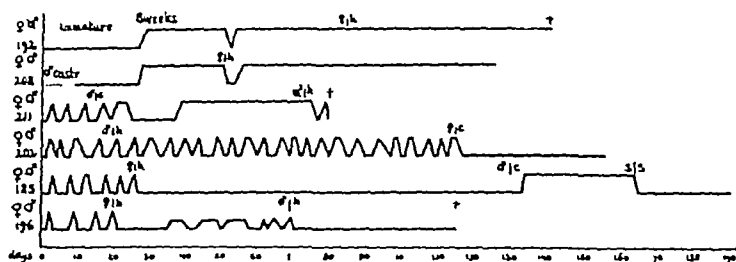


FIG 1

Selected oestrus curves of parabiotic pairs of rats. The base corresponds to the anoestrous and dioestrous condition, the top to full oestrus (stage 2-3). *Pair 192* male was castrated at birth, united with female at 3 weeks. The female, when 8 weeks old, starts oestrus which is constant with only one interruption of 2 days. Eight weeks later the female is hypophysectomized (♀ h) but remains in constant oestrus. *Pair 208* Adult male is castrated and united with adult female on same day. No smears taken for first 3 days. Constant oestrus established on 28th day. Female hypophysectomized on 51st day of experiment. *Pair 211* Normal pair, female has shown regular cycles for some time. Male castrated on 15th day. Female establishes constant oestrus on 39th day. Castrate hypophysectomized on 73d day. Female resumes normal cycles on 75th day. *Pair 202* Normal pair. Male hypophysectomized on 20th day. Female continues normal cycles until castration on 115th day. *Pair 185* Normal pair. Female hypophysectomized on 26th day, falls immediately into anoestrus. Castration of male on 130th day brings female into constant oestrus within 4 days. After separation from the castrate on the 164th day the female returns immediately into anoestrus (165th day). *Pair 196* Normal pair. Female hypophysectomized on 20th day, goes immediately into anoestrus but after the 35th day shows periods of incomplete oestrus. After hypophysectomy of the male complete anoestrus is established again.

Later, constant oestrus is maintained in all cases (Fig 1, pair 208).

The ovaries of these hypophysectomized females in constant oestrus are extremely enlarged and contain large numbers of growing follicles (Fig 2) some of excessive size, others equal to normal mature follicles. New follicles obviously are added constantly while the older ones degenerate (Fig 2, follicles of irregular shape). These ovaries are larger even than those of non-hypophysectomized females under similar conditions (Fig 6, Levine and Witschi<sup>3</sup>). The uteri are widely distended and their lumen is filled with cell-debris and leucocytes (Fig 3). In 4 cases the females were separated from the castrate males 5 or more weeks after hypophysectomy. Within 2 days they fell into anoestrus (compare Fig 1 pair 185 S,S). Ovaries preserved at different intervals show a rapid atresia of all mature follicles, without ovulation. It is evident therefore, that the follicular stimulation was due to a hormone received from the castrate.

That this hormone originates from the hypophysis of the castrate is definitely shown in a third set of experiments in which the castrate is hypophysectomized (2 cases), whereupon the unoperated

well as X-ray sterilized males and females (Levine and Witschi<sup>3</sup>) produce the same effects as castrates (Fig 6) if put in parabiosis with normal females. This is remarkable since the secondary sex characters are maintained in these cases. An histological examination of the hypophyses proves that the characteristic "castrate cells" are present not only in castrates but also in cryptorchids (Fig 7) and in X-rayed males and females (Levine and Witschi<sup>3</sup>). Destruction of the germ cells by either treatment causes similar or identical histological and functional changes in the hypophysis.

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In a first experiment it was attempted to unite females hypophysectomised for over one month with male castrates. This was not very successful. The females, which were weakened by hypophyseal deprivation, died in all but one case shortly after the operation. In the one surviving case the female recovered to a healthy condition only after several weeks. She remained in anoestrus during the first month of parabiosis and then went into constant oestrus. Obviously, the anoestral phase was due to the poor general condition of the female, following the parabiosis operation. The oestral phase, which has lasted now for 2 months with only one short interruption, must be due to the influ\ of hypophyseal hormones from the castrate.

A second experiment starts with females that had been in parabiosis with castrate males from 2 to 6 months. All had established the constant oestrus condition. In 5 pairs of this type the hypophysis of the female was removed with no effect upon the oestral type of vaginal smears (Fig 1, pair 192). It is true that some of the females fell once or twice into anoestrus for periods of 1 to 3 days, immediately following hypophysectomy, though apparently this was due merely to general disturbances caused by the operation.

<sup>3</sup> Levine, W. T., and Witschi, E., *Proc Soc Exp Biol and Med*, 1933, **30**,

1152

<sup>4</sup> Fels, E., *Arch f Gynaek*, 1929, **138**, 16

<sup>5</sup> Kallas, H., *Pflüg Arch ges Physiol*, 1929, **223**, 232

<sup>6</sup> Martins, Th., *Compt Rend Soc de Biol*, 1929, **103**, 1341

Fig 2

Parabiotic pair 186 Ovary of a hypophysectomized female, united with castrate male, after constant oestrus of  $5\frac{1}{2}$  months  $\times 12$

Fig 3

Parabiotic pair 210 Similar conditions as in previous case Cross section of uterus.  $\times 12$

Fig 4

Parabiotic pair 205 Ovary of a hypophysectomized female united with normal female Preserved after  $2\frac{1}{2}$  months of anoestrus  $\times 12$

Fig 5

Same pair as Fig 4 Uterus of hypophysectomized female  $\times 12$

Fig 6

Parabiotic pair 175 Ovary of female in parabiosis with X ray sterilized female Preserved after  $3\frac{1}{2}$  months of constant oestrus  $\times 12$

Fig 7

Parabiotic pair 178 Anterior lobe of hypophysis of the male twin, one-half year after experimental cryptorchidism had been established. Note the large "cast cells" filled with colloid.  $\times 200$

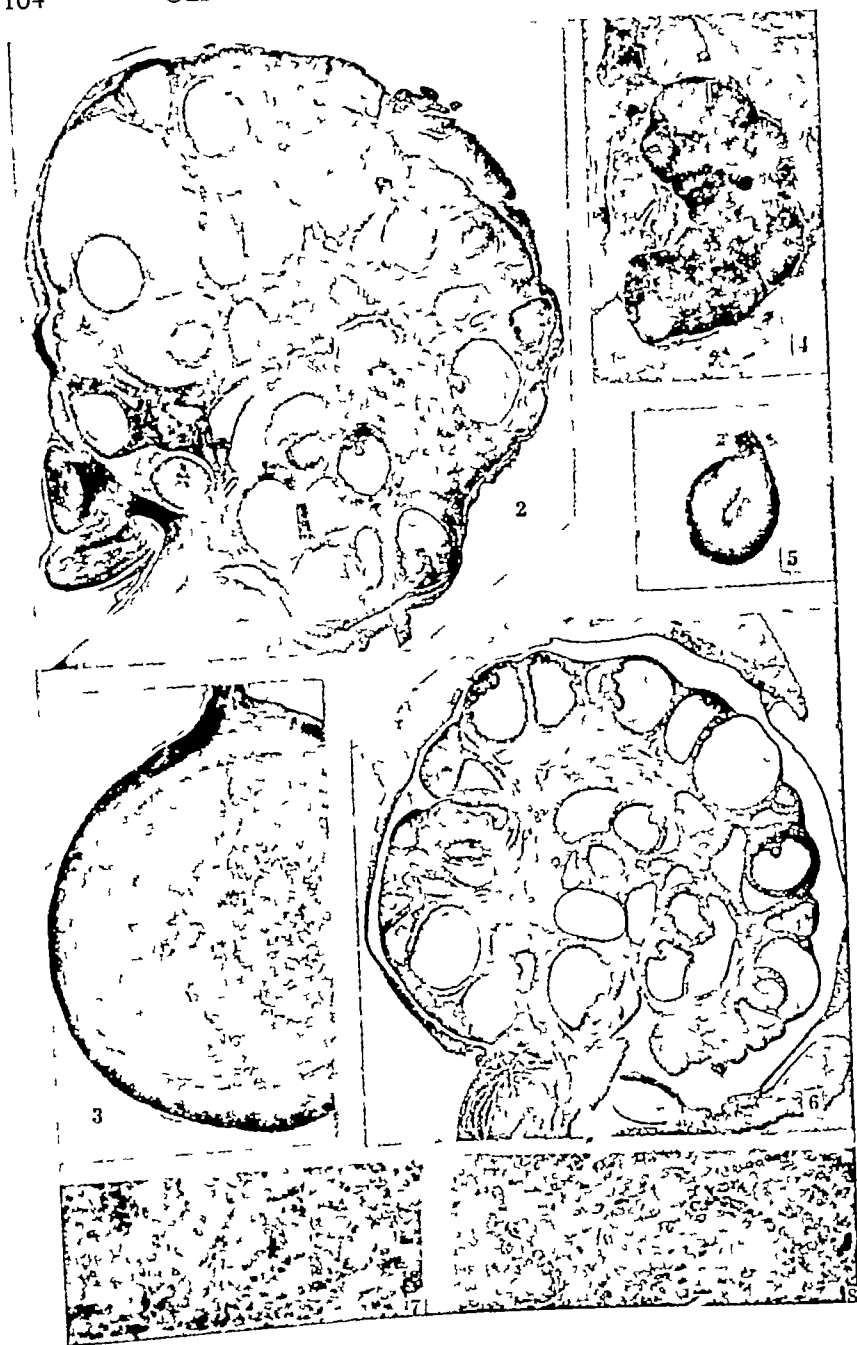
Fig 8

Same pair as Fig 7 Anterior lobe of hypophysis of the female twin, which had been in constant oestrus for more than 4 months Nearly normal histological picture  $\times 200$

female returns from the constant to cyclical oestrus (Fig 1, pair 211) Normal cycles are maintained also by females in parabiosis with hypophysectomised females or males (Fig 1, pair 202)

The reported experiments show clearly that the condition of constant oestrus is due to an influx of follicular growth stimulating hormone which is released in great quantities by the hypophysis of the castrate co-twin There remains to be ascertained where the luteinizing hormone comes from which causes the extensive formation of corpora lutea and the prolonged phases of anoestrus during the first period after castration The case 211 (Fig 1) may illustrate once more the sequence of typical reactions that follow upon castration of the male twin The female still runs one normal cycle, then a prolonged cycle and then falls into a period of anoestrus Constant oestrus is established only during the fourth week In more than 20 similar cases Hill (1 c) and the present authors have found that the period of irregularity preceding constant oestrus can last from 3 to 18 weeks So, evidently, the hypophyseal system which produces the luteinizing hormone subsists slowly under the influence of the continuous stream of follicle stimulating hormone Evidence that the hypophysis of the unoperated female is the source of the luteinizing hormone comes from a set of experiments in which we observe that constant oestrus follows castration of the male within 4 or 5 days if the female had been hypophysectomised 5 to 15 weeks previously This experiment (4 cases) runs in the following way

We start with normal pairs (Fig 1 pair 185) After normal cycles of the female had been observed for some time she is hypo-



hormone Furthermore we conclude that the maintenance in the blood stream of a constant and relatively high level of follicle stimulating hormone suppresses the production of luteinizing hormone by the hypophysis

## 7577 P

## Nervous Control of Thyroid Activity I Effect of Pilocarpin and Adrenalin on Metamorphic Action of Thyreoactivator

EDUARD UHLENHUTH, EDGAR VAN SLYKE AND KARL MECH

*From the University of Maryland Medical School \**

In the experiments reported here we have used amphibian metamorphosis (in the larvae of *Ambystoma tigrinum*) as a physiological sign of the effects of the thyroid hormone, pilocarpin and adrenalin as stimulators of the parasympathetic and sympathetic nervous system respectively, and injections of thyreoactivator from the anterior lobe of the beef hypophysis<sup>1-4</sup> as an activator of the thyroid function

When pilocarpin or adrenalin alone is injected intraperitoneally, no visible effect on metamorphosis is obtained It will be shown here that the injection of either of these drugs together with thyreoactivator increases the sensitivity of the larvae to the metamorphic action of the thyreoactivator

In one representative experiment (CCCLVI, 1933) a number of the larvae of the tiger salamander were divided into 4 groups Group "a", controls injected with Ringer solution, Group "b, c and d" received triweekly intraperitoneal injections of thyreoactivator extracted from approximately 30 mg dried anterior lobe, per animal and injection, group "c" received in addition triweekly injections of 2 mg pilocarpin-hydrochloride (Merck) per animal and injection, group "d" received in addition triweekly injections of 0.05 mg adrenalin-chloride, 1:1000 (Parke, Davis Company), per animal and injection

\* Aided by the Weaver Fellowship Fund and Julius Friedenwald Fund of the University of Maryland School of Medicine

<sup>1</sup> Uhlenhuth, E, *Anat Rec*, 1926, **34**, 119

<sup>2</sup> Uhlenhuth, E, and Schwartzbach, S, *Brit J Exp Biol*, 1927, **3**, 1

<sup>3</sup> Uhlenhuth, E, and Schwartzbach, S, *Proc. Soc. Exp. Biol. and Med.*, 1928, **26**, 149

<sup>4</sup> Uhlenhuth, E, and Schwartzbach, S, *Proc. Soc. Exp. Biol. and Med.*, 1928, **26**, 152

physectomised Immediately she falls into anoestrus, though in some cases she recovers partly, showing irregular and incomplete oestral changes in the vaginal smears That this activity is due to stimulation by the normal male parabiont can easily be demonstrated Subjecting him to hypophysectomy brings the female into permanent anoestrus at once (Fig 1, case 196) Even if the vaginal smears show constantly the anoestral condition, as in case 185, the ovaries are slightly larger than in single hypophysectomised females or in females of double-hypophysectomised pairs These ovaries contain large numbers of small egg follicles (white dots in Fig 4) which later undergo fibrous degeneration No luteinization is observed, and the output of oestrin must be negligible, as indicated by vaginal inactivity and reduced size of uteri (Fig 5) It now, after an anoestral period of 5 to 15 weeks the male parabiont is castrated, then the increased amount of hypophyseal hormones coming in from the castrate immediately causes a rapid growth of the Graafian follicles and constant oestrus is established within 4 or 5 days (Fig 1, case 185)

This quick and clear cut reaction of hypophysectomised as compared with normal females makes it quite certain that in the latter the mainly anoestral period preceding constant oestrus is due to the production of luteinizing hormones by the female hypophysis As long as this lasts, excessive corpus luteum formation will result because of the simultaneous stimulation of follicular growth by the castrate hypophysis However the maintenance of a constant and high concentration of follicle stimulating hormone in the blood stream tends to suppress the production and the release of the luteinizer Nevertheless the hypophysis of the female in constant oestrus retains the potency of producing the luteinizer For such females, if separated from their castrate twins, resume cyclical changes and may become pregnant and have litters again Histologically the hypophysis preserves the normal appearance even after months of constant oestrus (Fig 8) The fact that the two gonadotropic hormones of the hypophysis thus act as an antagonistic pair probably plays an important role in the establishment of normal oestrus cycles

*Conclusions* Our experiments with parabiotic rats corroborate the contention of Zondek<sup>1</sup>, and Hellbaum<sup>2</sup> gained from entirely different sources of evidence, that the castrate hypophysis emits an increased quantity of follicle stimulating hormone but no luteinizing

<sup>1</sup> Zondek, B, *Arch f Gynaek*, 1930, 144, 133

<sup>2</sup> Hellbaum, A, *Proc Soc Exp Biol and Med*, 1933, 30, 641

castrate females showed a progressive loss of weight while normal females maintained their weight

II Effect of estrin on sex organs A Male The testes in Series A weigh only 65%, those of Series B only 23% of the control weights In the animals of Series B no spermatids or spermatozoa were found, and the tubules contained no secondary, and only a few primary spermatocytes However, numerous mitotic figures were present in the spermatagonia The interstitial tissue was considerably reduced The last breeding in which females were impregnated with these males occurred 19 days after commencement of the injections

B Female The ovaries in Series A are 66% and those of Series B 28% of the control weights The uterus of the normal female showed a progressive increase in diameter and weight The uterus of the castrate was restored to approximately the size of the normal female There was, however, considerable variation in size of the individual uteri and the measurements are not entirely accurate because of contractions and consequent thickening The weight cannot be used for comparison in this group because at ovariectomy a variable length of the uterine horn had been removed along with the ovary

III Effect of estrin upon the hypophysis In the male and castrate female of Series B the hypophysis is approximately 100% heavier than that of the control weights In the normal female the hypophysis is about 200% heavier than the control

Histologically, the pituitaries of females who were ovariectomized 8 months before and injected with estrin for 8 weeks contain very few castration cells These cells never equalled in size the large "signet ring" cells seen in the untreated animal

In the anterior pituitaries of normal males and females that received estrin there was an increase in the number of chromophobe cells with a corresponding increase in the number of cells transitional between chromophobes and basophiles Both the "transitional" cells and the basophiles contain a markedly hypertrophied Golgi apparatus and nucleolus, and numerous enlarged mitochondria, all of which suggest abnormally secreting cells Many "degranulated" basophiles are to be noted The connective tissue and vascular elements are also increased

The pituitaries of Series A resemble somewhat the pituitary of pregnant animals confirming Baniecki's<sup>5</sup> observations on the guinea pig

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<sup>5</sup> Baniecki, H., *Arch f Gynak*, 1928, 134, 693

Group "a" remained larval for the period of the experiment, Group "b" needed an average of 41 injections of thyreoactivator to metamorphose, Group "c" (pilocarpin) needed an average of 12 injections of thyreoactivator and Group "d" (adrenalin) an average of 20 injections of thyreoactivator

Further work is in progress to show whether the sensitizing effect of pilocarpin and adrenalin is due to a stimulation of specific thyroid secretory nerves or to other effects

## 7578 P

## Effects of Estrin upon Gonads, Mammary Glands and Hypophysis of the Rat \*

S R HALPERN AND F E D'AMOUR (Introduced by I E Wallin)

From the Department of Anatomy, School of Medicine, University of Colorado, and Research Laboratories, University of Denver

It is generally agreed that injections of estrin cause an atrophy of the gonads<sup>1</sup> and an increase in the weight of the hypophysis with a decrease in its gonad-stimulating power<sup>2</sup> Wade and Doisy,<sup>3</sup> however, state that Theelin, in dosages up to 66 gamma daily, in the male does not cause an interruption of spermatogenesis, and in the female similar doses do not interfere with normal reproductive processes

Adult normal males and females and female castrates were divided into 2 series A and B In Series A, 5 R U of estrin<sup>4</sup> were given daily for 3 weeks and 20 R U daily the fourth week In Series B the same dosage was given as in Series A but injections of 20 R U daily were continued for 4 weeks more Each series included 3 groups with 10 rats in each group 1, normal males, 2, normal females, and 3, ovariectomized females An adequate number of controls was used

### Results I Effect of estrin on body weight Normal males and

\* This investigation was aided in part by a grant from the National Research Council, Committee on Problems in Relation to Sex

<sup>1</sup> Moore, C R., and Price, D, *Am J Anat*, 1932, 50, 13

<sup>2</sup> Leonard, S L, Meyer, R K., and Hsaw, F L, *Endocrinology*, 1931, 13, 17

<sup>3</sup> Wade, N J., and Doisy, E A, *Abst Proc Am Fed Biologists*, 1934

<sup>4</sup> Estrin prepared from pregnancy urine according to the method of D'Amour, F E., and Gustavson, R G., *J Pharm and Exp Therap*, 1930, 40, 4



castrate females showed a progressive loss of weight while normal females maintained their weight

II Effect of estrin on sex organs A Male The testes in Series A weigh only 65%, those of Series B only 23% of the control weights In the animals of Series B no spermatids or spermatozoa were found, and the tubules contained no secondary, and only a few primary spermatocytes However, numerous mitotic figures were present in the spermatagonia The interstitial tissue was considerably reduced The last breeding in which females were impregnated with these males occurred 19 days after commencement of the injections

B Female The ovaries in Series A are 66% and those of Series B 28% of the control weights The uterus of the normal female showed a progressive increase in diameter and weight The uterus of the castrate was restored to approximately the size of the normal female There was, however, considerable variation in size of the individual uteri and the measurements are not entirely accurate because of contractions and consequent thickening The weight cannot be used for comparison in this group because at ovariectomy a variable length of the uterine horn had been removed along with the ovary

III Effect of estrin upon the hypophysis In the male and castrate female of Series B the hypophysis is approximately 100% heavier than that of the control weights In the normal female the hypophysis is about 200% heavier than the control

Histologically, the pituitaries of females who were ovariectomized 8 months before and injected with estrin for 8 weeks contain very few castration cells These cells never equalled in size the large "signet ring" cells seen in the untreated animal

In the anterior pituitaries of normal males and females that received estrin there was an increase in the number of chromophobe cells with a corresponding increase in the number of cells transitional between chromophobes and basophiles Both the "transitional" cells and the basophiles contain a markedly hypertrophied Golgi apparatus and nucleolus, and numerous enlarged mitochondria, all of which suggest abnormally secreting cells Many "degranulated" basophiles are to be noted The connective tissue and vascular elements are also increased

The pituitaries of Series A resemble somewhat the pituitary of pregnant animals confirming Bamecki's<sup>5</sup> observations on the guinea pig

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<sup>5</sup> Bamecki, H., *Arch f Gynak*, 1928, 134, 693

All animals in Series B possessed actively secreting mammary glands. The extent of this development as far as the size of the gland is concerned was greatest in the males, somewhat less in the ovariectomized females, and least in the non-castrate females. A white, fatty fluid (milk?) flowed freely from cut portions of these glands. Histological examination showed a flattened epithelium, prominent vacuolization and alveoli distended with fluid containing many fat droplets and numerous free vacuolated cells.

The effect of long continued injections of estrin upon the mammary glands and hypophysis suggest that the hypophysis is functioning abnormally. Whether this lactation is the result of estrin administration *per se* or whether it resulted after the cessation of the injections is not known, as the animals were sacrificed 4 days after the last injections. This question is now being investigated.

## 7579 C

## Fibrinolytic Activity of Hemolytic Streptococci on Blood of Cases of Recurrent Tropical Lymphangitis

P. MORALES OTERO AND A. POMALES LEBRON (Introduced by A. M. Pappenheimer)

*From the School of Tropical Medicine, University of Puerto Rico, under the auspices of Columbia University*

Tillett and Garner<sup>1</sup> have recently shown that broth cultures of hemolytic streptococci of human origin rapidly dissolve normal human fibrin clot. Tillett, Edwards and Garner<sup>2</sup> demonstrated the development of resistance to dissolution in the plasma clot obtained from individuals following acute hemolytic streptococcus infections. They also showed that this antifibrinolytic property is absent in the fibrin clot derived from a group of patients convalescing from other infections. Likewise, the blood from the great majority of healthy adults and from persons with other acute diseases was found to be susceptible to fibrinolysis. The authors believe that this insusceptibility to dissolution is specifically induced and that "the fibrinolysin of hemolytic streptococci in the body makes a definite response directed against the lytic action of the bacteria."

While studying the probable relationship of hemolytic strepto-

<sup>1</sup> Tillett, W. S., and Garner, R. L., *J. Exp. Med.*, 1933, **58**, 485

<sup>2</sup> Tillett, W. S., Edwards, L. B., and Garner, R. L., *J. Clin. Invest.*, 1934, **12**, 47

cocci to recurrent tropical lymphangitis, we have made a series of fibrinolytic tests with the blood of a group of patients suffering from this condition. In performing the test the method employed was that recommended by Tillett, Edwards and Garner<sup>2</sup> with the exception that we used tryptic digest broth instead of glucose broth to grow the streptococci.

Thirty-three strains of hemolytic streptococci isolated from different conditions were tested for their fibrinolytic activity on the plasma of a normal individual. All determinations were made at the same time, by the same person, and under similar conditions. The results are shown in Table I.

TABLE I.

Fibrinolytic Activity of Strains of Hemolytic Streptococci from Different Sources

Strain No	Source	Complete dissolution in hrs
*N Y <sub>5</sub>	Scarlet fever	None†
M <sub>4</sub>	Sore throat	4 hr
S <sub>2</sub>	Septicemia	8 "
*T	Tonsils	2 "
M <sub>5</sub>	Sore throat	5½ "
S <sub>1</sub>	Septicemia	3½ "
T <sub>5</sub>	Tonsils	3½ "
O <sub>1</sub>	Osteomyelitis	2¼ "
T <sub>1</sub>	Tonsils	12 "
T <sub>5</sub>	"	2¼ "
A <sub>10</sub>	Meninges	2¼ "
A <sub>8</sub>	Abscess	5½ "
A <sub>3</sub>	Lesion on knee joint	2½ "
A <sub>4</sub>	Pustule	4 "
T <sub>0</sub>	Tonsils	5 "
T <sub>3</sub>	"	8 "
M <sub>2</sub>	Sore throat	20 "
T <sub>8</sub>	Tonsils	2½ "
T <sub>10</sub>	"	2½ "
A <sub>5</sub>	Deep chronic lesion on face	1¼ "
T <sub>4</sub>	Tonsils	10 "
T <sub>11</sub>	"	4 "
A <sub>11</sub>	Pustule	2 "
*E	Erysipelas	2½ "
L <sub>14</sub>	Lymphangitis	1 "
L <sub>6</sub>	"	1 "
L <sub>12</sub>	"	1¼ "
L <sub>1</sub>	"	5 "
A <sub>12</sub>	Impetigo lesion on leg	3 "
L <sub>7</sub>	Lymphangitis	3½ "
L <sub>9</sub>	"	Partial dissolution in 24 hr
L <sub>11</sub>	"	2 hr
L <sub>8</sub>	"	4½ "

\*Kindly sent to us by Dr. A. F. Coburn.

†None in 24 hrs

From these, 2 strains, Nos. T<sub>8</sub> and E, were selected for carrying out the fibrinolytic determinations. The results are shown in Table II.

TABLE II  
Fibrinolytic Determinations

Case	Condition	Strains	
		E <sub>1</sub>	T <sub>8</sub>
		Complete dissolution in	
A P	Normal	1 hr	2 hr
M E M	"	$\frac{2}{3}$ "	$\frac{2}{3}$ "
L G	"	$1\frac{1}{4}$ "	$\frac{2}{3}$ "
E R	"	1 "	$\frac{2}{3}$ "
J M	"	$\frac{2}{3}$ "	$\frac{2}{3}$ "
M N	"	$\frac{1}{2}$ "	$\frac{2}{3}$ "
T V	"	2 "	$\frac{2}{3}$ "
G V	Typhoid	3 $\frac{1}{2}$ "	$1\frac{1}{4}$ "
C A	"	1 $\frac{1}{4}$ "	$1\frac{1}{4}$ "
A S	"	Clot retraction	$1\frac{1}{2}$ "
A C	"	Nonet	7 "
N C	Thrombophlebitis	2 $\frac{1}{2}$ hr	$1\frac{1}{4}$ "
J G	Common cold	2 "	$1\frac{1}{4}$ "
Z C	R lymphangitis	12 "	$\frac{2}{3}$ "
R A	Lymphangitis	Nonet	Nonet
J L	R lymphangitis	Nonet	Nonet
M R	"	14 hr	8 hr
P L	"	Nonet	Nonet
M R	"	6 $\frac{1}{2}$ hr	22 hr
P L	Lymphangitis	Nonet	Nonet
M R	R lymphangitis	Nonet	Nonet
F J	"	Nonet	Nonet
J C *	"	$\frac{1}{4}$ hr	$\frac{2}{3}$ "
J P *	"	$\frac{1}{2}$ "	$\frac{1}{2}$ "
F M	R lymphadenitis and lymphangitis	Nonet	Nonet

\*In these 2 cases, pure cultures of hemolytic streptococci were isolated from local lesions at the time determinations reported were made

†None in 24 hrs

Repeated determinations at varying intervals were made in several cases of recurrent tropical lymphangitis. In 4 cases the plasma clot exhibited maximum resistance from the onset of the acute attack to 6 weeks after the attack. In one case, the dissolution time was 14 hours, 2 hours after the onset of symptoms, and maximum resistance on the 18th and 59th day after the attack. Another case exhibited maximum resistance when the attack was subsiding and showed complete dissolution in 10 hours, 8 days after the attack, and in 8 hours, 24 days after the attack.

In 2 cases in which virulent hemolytic streptococci were isolated from local lesions in the affected limb during the acute attack, fibrinolysis was complete in 30 minutes during the attack, and in 2 hours, 8 days after the attack.

In a normal control, where repeated determinations were made at short intervals during 2 months the dissolution time varied slightly from 30 minutes, the lowest, to 1 hour and 30 minutes, the highest

*Summary* Fibrinolytic determinations made with 33 strains of hemolytic streptococci isolated from different conditions showed individual variations in their lytic activity when tested under similar conditions. Apparently, the plasma clot derived from cases of recurrent tropical lymphangitis develops a definite resistance to the fibrinolytic activity of hemolytic streptococci.

## 7580 C

## Refinements in X-ray Technique for the Estimation of Vitamin D \*

BRIAN O'BRIEN AND KENNETH MORGAREIDGE (Introduced by W. R. Bloor)

*From the University of Rochester, Rochester, New York*

None of the existing techniques commonly employed for the estimation of Vitamin D are entirely beyond criticism. In choosing the most suitable procedure a number of factors must be considered, depending upon whether time and economy or accuracy and reliability are to receive the most consideration. To indicate briefly some of these factors, we have but to point out the principal advantages and limitations of the 3 widely used methods for the assay of antirachitic potency, in all of which inbred stocks of albino rats furnish the experimental animals.

Since all 3 methods may be adapted to either curative or preventive procedures and since the former enjoy by far the greater vogue in this country, the present discussion and experimental work are limited to curative methods. The Steenbock diet No. 2965 was used to produce rickets, and, in general, the recommendations of the Committee of the American Drug Manufacturers Association on Vitamin Assay were followed.<sup>3</sup>

The bone-ash technique as worked out principally by Chick, Roscoe and others<sup>1, 2</sup> is a purely objective procedure, not subject to aberrations of human judgment, even to the extent that they occur in the other 2 methods. On the other hand, the factor of biological variation exerts its greatest influence in this method, and the statis-

\* We wish to express our thanks to Dr. Stafford L. Warren and the Department of Radiology of Strong Memorial Hospital for suggestions and the use of X-ray equipment and also to the Department of Biochemistry for animal facilities.

<sup>1</sup> Chick and Roscoe, *Biochem. J.*, 1926, **20**, 137.

<sup>2</sup> Chick, Korenchevsky, and Roscoe, *Biochem. J.*, 1926, **20**, 622.

<sup>3</sup> Holmes, *Rep. A. D. M. A., Com. Vit. Assay*, 1932.

tical significance of the results can be increased only by proportionally lengthening the series of experimental observations in order that the average figures may not be unduly influenced by such factors as variation in animal stature, susceptibility to rickets, etc

The "line-test" furnishes the most convenient method from the standpoint of time and equipment and can be brought to a high degree of dependability<sup>3</sup> Here also, however, animal variation may markedly influence the results, particularly variations in susceptibility to the effects of the rachitogenic diet The only check possible on this point is that obtained by preliminary tests done on random animals before the beginning of the experimental period Any adequate line-test procedure includes photographing the specimens as soon as the "line" has been developed in order that permanent records may be obtained<sup>4</sup> A convenient camera for this work was constructed For the tibia, the negative is made at unit magnification on fine grain film Prints may be made at magnifications up to 10 diameters without film grain becoming noticeable



FIG 1  
Rat and film holder with lead shields

<sup>3</sup> McCollum, Simmonds, Shipley and Park, *J Biol Chem*, 1922, 51, 41

<sup>4</sup> Stevens and Nelson, *Ind Eng Chem Anal ed*, 1932, 4, 200

One of the first X-ray techniques at all comparable to the line-test was suggested by Poulsson and Lovenskiöld<sup>5</sup>. The idea has been further elaborated by Bourdillon, et al<sup>6</sup>. Since X-ray photographs of each individual animal are taken before the beginning of the experimental period, anomalous cases can be recognized and the chief objection to the other 2 methods is overcome, in that variations in susceptibility to the rickets-producing diet do not weight the final results. One serious objection to these techniques, however lies in the fact that only 2 X-rays are taken, one at the beginning of the experimental period, under anesthesia, and the other at the end of the test after the animals have been killed. While more frequent radiography is desirable, increasing the amount of anesthesia is to be avoided as it may frequently lead to increased incidence of respiratory infection and other metabolic upsets among the experimental animals.

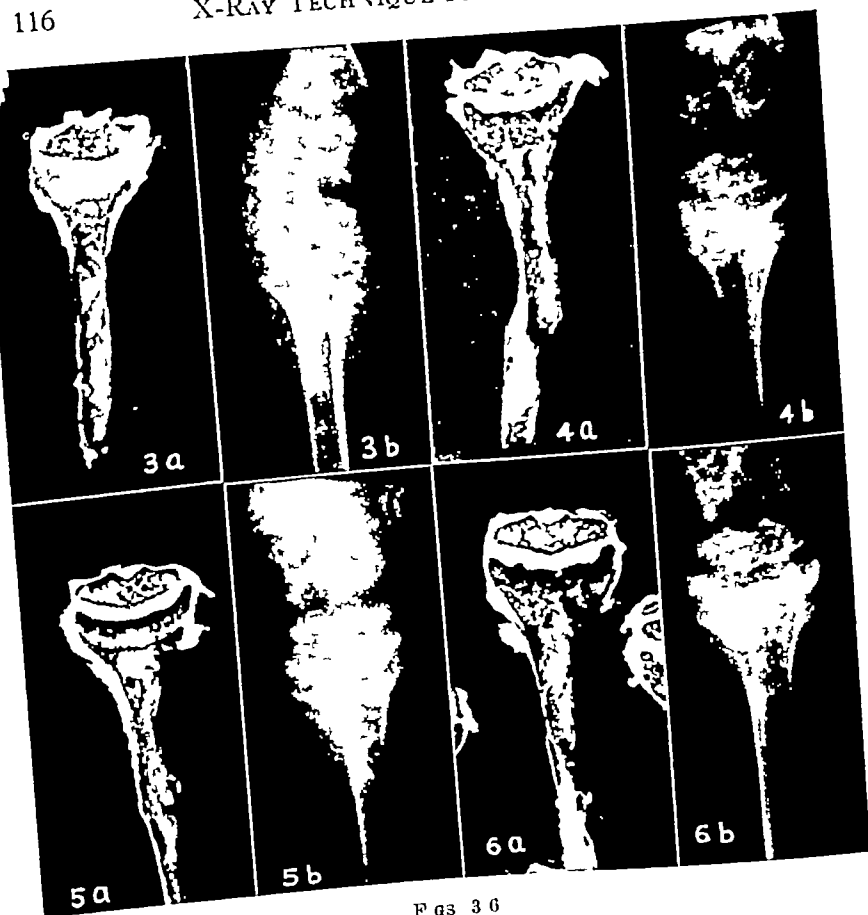
To overcome this difficulty, and to avoid the use of strenuous methods of clamping the rats in position for photographing, which often results in injury, the present modification was developed. Duplitized dental film was held in a special holder (Fig 1) of such dimensions and so shielded with sheet lead as to allow both fore and hind legs of the rats to be held in place for exposure with-



FIG 2  
Method of holding rat for X ray photograph of tibial head

<sup>5</sup> Poulsson and Lovenskiöld, *Biochem J*, 1928, **22**, 135

<sup>6</sup> Bourdillon, Bruce, Fischmann and Webster, Med. Res. Council Special Report Series No 153



Figs 3 &amp; 6

- 3 a, b Line test and X ray of rachitic rat  
 4 a, b Line test and X ray of healed rickets Total dose, 16 international units  
 in form of irradiated milk  
 5 a, b Line-test and X ray showing effect of total dose of 9 units of international standard solution of vitamin D  
 6 a, b Line-test and X ray showing effect of total dose of 10 international units in form of irradiated milk

out endangering the hands of the operator (Fig 2) The dental packets were placed in a slide and pushed against a stop Suitable clips were provided to hold lead stencil numbers for identification With a little practice, the rats may be held quite easily and loosely without showing any of the fine muscular tremor which accompanies mechanical clamping There was very little spoilage of film

We feel that the advantages accruing from the X-ray estimation of Vitamin D by the present technique are obvious Each animal is checked before the beginning of the test feeding period to estab-



lish the degree of rickets. The progress of healing is followed in each animal at frequent intervals so that any anomalies are recognized. Permanent records are provided and may be re-read at any time. While subjective, the estimations, if based on a comparison of the unknown antirachitic substance with a standard, provide a completely null method of observation. Thus, different observers comparing the X-rays may be expected to arrive at similar results. The averages compare closely with either line-test or bone-ash averages on the same animals, and the spread of observations is certainly no greater than that found with the other methods.

A comparison was made between line-test, X-ray and bone ash estimations on a small series of animals (18 in all) used to determine the potency of an irradiated milk in terms of the International Standard Vitamin D solution. The results (Table I) represent independent estimations by the 3 methods uninfluenced by the other 2. It is felt that the agreement is very good considering the few animals used.

TABLE I

Feeding Level International Units per day	International Units per cc of Milk		
	Bone Ash	Line Test	X Ray
0.5	0.50	0.40	0.40
1.0	0.45	0.40	0.40
2.0	0.50	0.53	0.50
Average	0.48	0.44	0.43

We are indebted to Dr. E. M. Luce-Clausen, who very kindly made the bone ash determinations reported here.

It seems to have been generally recognized among workers in the field, although not, to our knowledge, specifically pointed out in the literature, that the nature of the calcification is by no means independent of the type of antirachitic substance fed. In particular, when an irradiated milk is compared with an irradiated ergosterol concentrate, it has been noticed that the initial calcification is frequently more diffuse in character, "a narrow and continuous line of calcification" seldom occurring in the milk-fed animals.

Examples of the line-test and X-ray photographs on the same animals are shown in Figs. 3 to 6 at equal magnification. Not only the degree but also the character of the healing is shown. In Fig. 5, the narrow line of calcification (animal fed International Standard irradiated ergosterol solution) is shown in marked contrast to the diffuse calcification occurring in an animal fed irradiated milk at substantially the same potency level, Fig. 6.

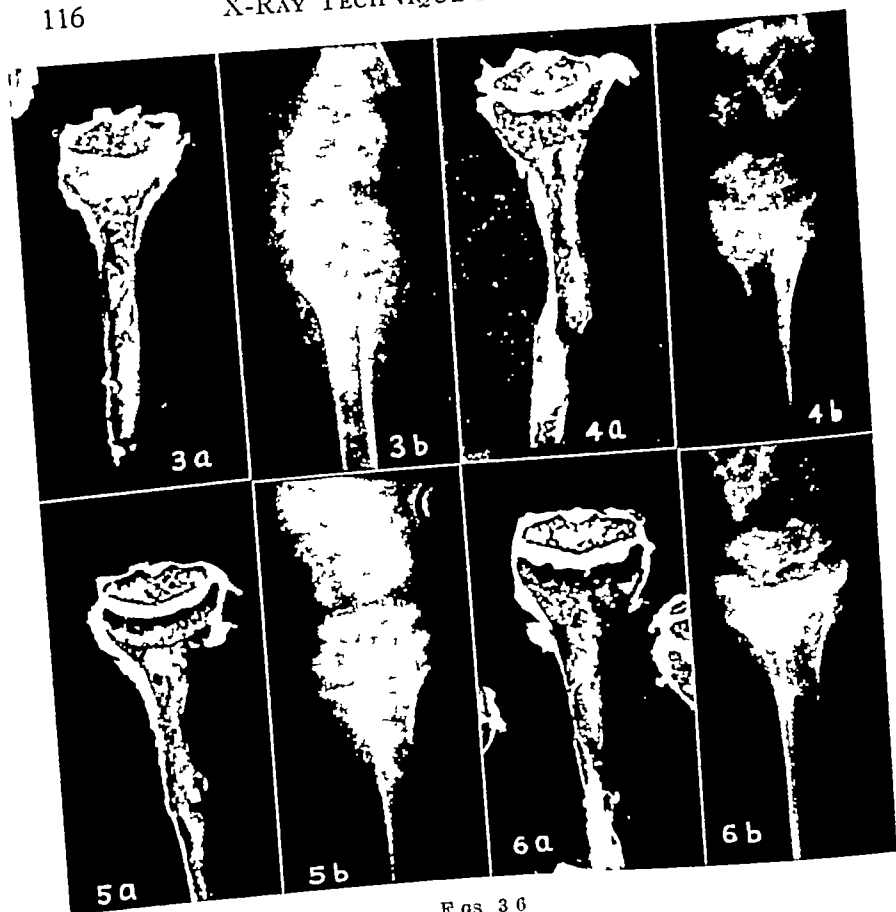


FIG 3 6

- 3 a, b Line test and X ray of rachitic rat  
 4 a, b Line test and X ray of healed rickets Total dose, 16 international units  
 in form of irradiated milk  
 5 a, b Line test and X ray showing effect of total dose of 9 units of interna  
 tional standard solution of vitamin D  
 6 a, b Line test and X ray showing effect of total dose of 10 international  
 units in form of irradiated milk

out endangering the hands of the operator (Fig 2) The dental packets were placed in a slide and pushed against a stop Suitable clips were provided to hold lead stencil numbers for identification With a little practice, the rats may be held quite easily and loosely without showing any of the fine muscular tremor which accompanies mechanical clamping There was very little spoilage of film We feel that the advantages accruing from the X-ray estimation of Vitamin D by the present technique are obvious Each animal is checked before the beginning of the test feeding period to estab-

scopic technic The result was quite surprising when 1 2 - 1 8 dilutions of the immune serum were added Immediate total agglutination occurred and in an hour all of the bacilli were so firmly attached to each other in a transparent disc, that they could not be separated by intensive shaking Higher dilutions of the immune serum (1 16-1 256) caused a typical floccular agglutination This agglutination was strictly specific No trace of agglutination occurred even when undiluted normal serum was added to the bacterial suspension.

Considering the peculiar nature of the agglutination, we had to weigh the possibility that we might be confronted with a special case of "agglutination by precipitin"<sup>3</sup> This suspicion seemed to us acceptable since we observed that a considerable quantity of P specific substance and a smaller quantity of C substance might be in solution after 24 hours in a broth culture of the encapsulated anthrax bacillus Consequently we carried out a few agglutination tests with bacilli washed carefully before the performance of the test No change occurred in the previously recorded results This reaction therefore had to be regarded as a true agglutination.

*The relation of precipitating antibodies to the agglutination* Our previous experiments concerning the antigenic structure of anthrax bacilli had made it very likely that the agglutination of the encapsulated anthrax bacillus had been caused by the same antibody which precipitated the P substance Direct experiments had to be carried out to prove this supposition

There was no difficulty in forming an opinion about the role of the C antibody since the commercial precipitating anthrax immune serum contains this antibody alone Four sera were used in this experiment, each of which gave specific precipitation with anthrax polysaccharide up to 1 1,000,000 dilution of the latter and no precipitation whatsoever with P substance No trace of agglutination was observed by using any of these sera in 1 1-1 1024 dilutions An entirely negative result was observed likewise by performing this test with a commercial protective immune serum, which contained none of the precipitating antibodies

On the other hand positive agglutination was obtained with each of the 5 immune sera prepared by us in rabbits through immunization with encapsulated anthrax bacilli Each of these sera contained an anti C immune body and gave at the same time precipitation with 1 500 000-1 000 000 dilutions of the P substance Furthermore a certain correlation could be demonstrated between the P precipitin

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<sup>3</sup> Jones, F S, *J Exp Med*, 1927, 46, 303, 1928, 48, 183

## Agglutination of the Encapsulated Anthrax Bacilli.

J TOMCSIK AND G BODON

*From the Department of Hygiene, Francis Joseph University, Szeged, Hungary*

There is no convincing experimental evidence on the specific agglutination of anthrax bacilli. The rapid sedimentation of most strains of anthrax bacilli makes a systematic study somewhat difficult. We know however that although by using certain special strains a stable suspension can be secured, the addition of immune serum will not be followed by any particulation, in spite of its containing a high concentration of precipitating antibodies.

A way was opened for the reinvestigation of this question since Szongott and one of us had discovered in the anthrax serum a highly active antibody, hitherto unknown<sup>1</sup>. The antibody of the anthrax immune serum known up to now and used for the thermoprecipitation test acts on the somatic substance of the bacillus, which is a polysaccharide. According to these authors, another and a more potent antibody is obtained, when suitable strains are used for immunization. This antibody reacts probably with the capsular material which is a carbohydrate-free, proteinlike substance. The 2 antibodies were named anti C and anti P respectively.

An accidental observation led us to study the effect of the anti P immune body on the agglutination of anthrax bacilli. We had primarily intended to study the phagocytosis of the encapsulated anthrax bacilli *in vitro*. The strain used for this experiment was isolated of the so called "Carbozoo" vaccine prepared by Mazzocchi and utilized in Italy for prophylactic immunization of animals<sup>2</sup>. This strain was virulent for laboratory animals, it could easily be emulsified in physiologic salt solution and produced in 24 hours an abundant capsule when cultured on agar at 37° C. In this respect this strain appeared very much similar to the "mucoid" varieties, which are obtained when virulent anthrax bacilli are attenuated by repeated subcultures at 41° C according to Pasteur's well known procedure. When the phagocytosis of this strain was studied in the presence of an immune serum containing both C and P antibodies, a very marked agglutination could be observed under the microscope.

The agglutination was then performed following the usual micro

<sup>1</sup> Tomcsik, J, and Szongott, H, *Z f Immunitätsforsch*, 1933, 77, 86

<sup>2</sup> Mazzocchi, M, *La Clinica Veterinaria*, 1931, 9, 3

the long chains. The agglutination by anti P immune serum was without exception positive whenever capsule formation could be revealed by microscopic study. The difference between the agglutination of the virulent strains and the attenuated "mucoid" varieties was that the former gave a floccular type of agglutination, whereas the latter, when lower dilutions of the serum had been used, was agglutinated in the characteristic disc form described above.

We confine ourselves to the description of one strain in order to illustrate the behaviour of the virulent and encapsulated anthrax bacillus. Anthrax strain No. 4 was isolated 6 years ago from naturally infected sheep and kept on agar medium with monthly subcultures. During this time it was transferred only a few times in mice. It was virulent for rabbits. At the time of our study the capsule production of this strain on agar medium was distinct. Its 24 hour agar growth consisted of long chains, some of these completely surrounded by a sharply defined capsule, twice as thick as the bacillary body. Other chains in turn had but a few capsulated members with well defined or with partly dissolved capsules. Some were entirely bare. At this stage this strain gave total agglutination with anti P immune serum up to a dilution of 1:512. The agglutination had a floccular character and contrary to the behaviour of the mucoid strains some inhibition was observed in the lower serum dilutions (up to 1:8). It was then subcultured daily for 15 days. At the end of this period it ceased to produce any capsule on agar medium and its agglutinability was completely lost, that is neither the P nor the C antibody produced any agglutination when mixed to this subculture.

*Summary* 1. Antianthrax immune serum containing both P and C precipitin agglutinates only the encapsulated anthrax bacilli. 2. Antianthrax immune serum containing only C precipitin does not give agglutination with any type of anthrax bacilli. 3. P antibody can be regarded as the anticapsular antibody. 4. The serological specificity of the capsules of attenuated "mucoid" strains is the same as that of the virulent anthrax bacillus, i.e. the latter produces capsules on culture media.

titer and the agglutinin titer of these sera. The agglutinin titer of the immune sera varied, however, according to the strain used in the experiment.

It was found but once, that the serum of a rabbit bled before the end of full immunization showed agglutination up to a dilution of 1:16, without giving any precipitation with the P substance. The lack of correlation was however temporary since the serum of the same rabbit gave both precipitation and agglutination after 2 more injections. This is another instance that during the immunization the agglutinating property of the immune serum manifests itself earlier than its precipitating property, owing to the fact that different proportions of antibodies are necessary to bring forward the appearance of a visible reaction in the 2 tests.

*Agglutination and capsule formation.* Two sera were selected for further experiments in order to study the correlation of capsule formation and of the agglutinability in different strains. One of the sera possessed a high anti C and no anti P precipitating activity, the other a high anti P and a lower anti C activity.

Ten different anthrax strains were selected for the first part of these experiments. It was carefully established in wet India ink preparations that none of these strains grown on ordinary agar media ever exhibited a trace of capsule formation. Most of these were virulent, a few attenuated. The bacterial suspensions were prepared by emulsifying a 24 hour agar growth in saline solution and shaking them for varying length of time according to their tendency for sedimentation. In most cases a homogeneous suspension could be obtained though the rate of sedimentation was sometimes increased. The results of the agglutination tests were completely negative, neither of the 2 immune sera caused any agglutination.

An attempt was also made to sensitize these strains through absorption of purified P substance. The bacteria washed and resuspended after this procedure however were just as refractory to forward agglutination as before.

On the contrary positive agglutination test was obtained with each of those strains which produced capsules on agar medium. We should emphasize here that not alone strains isolated of anthrax vaccines, or attenuated in our laboratory according to Pasteur's procedure belonged to this group. Almost 50% of our virulent old laboratory strains and some of the recently isolated fresh strains showed some capsule formation. It is true, that in many instances the capsule formation could be detected only by careful search in India ink preparations and extended but to one portion of some of

the long chains. The agglutination by anti P immune serum was without exception positive whenever capsule formation could be revealed by microscopic study. The difference between the agglutination of the virulent strains and the attenuated "mucoid" varieties was that the former gave a floccular type of agglutination, whereas the latter, when lower dilutions of the serum had been used, was agglutinated in the characteristic disc form described above.

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## Effect of Specific Antibody on the Capsule of Anthrax Bacilli

G BODON AND J TOMCSIK.

*From the Department of Hygiene, Francis Joseph University, Szeged, Hungary*

The characteristic effect exerted by the type specific immune serum on the capsule of pneumococci, a phenomenon observed by Neufeld, induced several investigators (Sabin,<sup>1</sup> Armstrong<sup>2</sup> and Logan and Smeall<sup>3</sup>) to work out a new rapid method for typing pneumococci. Apart from its practical importance this reaction is of theoretical interest, since it contributes direct proof to the previous supposition that the antipneumococcus immune sera induce in a specific way the alteration of the bacterial capsule. Tulczynska<sup>4</sup> demonstrated that this phenomenon occurs also in case of other encapsulated bacteria, pneumobacillus and streptococcus. Similar experiments performed by her with anthrax bacilli gave however very inconclusive results.

It seems somewhat striking that whereas studies on anthrax contributed perhaps most to the elucidation of the basic facts of immunity, and that the relation of capsule formation to bacterial virulence was first demonstrated with this microorganism, yet our present knowledge on the effect of immune serum on the capsule of the anthrax bacilli is considerably less than in many other bacteria.

Our previous work<sup>5</sup> revealed the existence of an antibody produced by immunization with encapsulated anthrax bacilli and probably acting on the bacterial capsule.

The strain used for the major part of our study produced abundant capsule on agar media. Its 24 hours' growth was very moist and sticky and it killed rabbits with typical symptoms of anthrax infection when a 1/1000 part of a 24 hour agar slant culture was injected subcutaneously. The strain was isolated from the "Carbozoo" vaccine.<sup>6</sup>

Fig 1 represents a wet India ink preparation showing that almost all of the bacilli are surrounded by well developed capsules.

<sup>1</sup> Sabin, *J Inf Dis*, 1930, **10**, 469

<sup>2</sup> Armstrong, R R, *Brit Med J*, 1931, **1**, 214, 1932, **1**, 187

<sup>3</sup> Logan and Smeall, *Brit Med J*, 1932, **1**, 188

<sup>4</sup> Tulczynska, R. E, *Z f Hyg u. Inf*, 1933, **114**, 760

<sup>5</sup> Tomcsik, J, and Szongott, H, *Z f Immunitätsforsch*, 1933, **77**, 86

<sup>6</sup> Tomcsik, J, and Bodon, G, *Proc Soc Exp Biol and Med*, 1934, **32**, 118

<sup>7</sup> Mazzucchi, M, *La Clinica Veterinaria*, 1931, **0**, 3





FIG 1

No trace of capsule was observed however, when the bacteria were suspended in physiologic salt solution and examined in hanging drop. The result did not differ, when one loopful of different dyes was mixed with the suspension. These observations conform with our previous knowledge, that the capsule of the anthrax bacillus is not visible in hanging drop because its refraction is similar to that of the surrounding fluid.

One loopful of different sera was then added to the bacterial suspension. Several normal as well as non-specific immune sera have been tried, but none of them caused any change in the microscopic picture. The anthrax sera studied here were divided again in 2 groups (1) sera containing only antipolysaccharide precipitins and

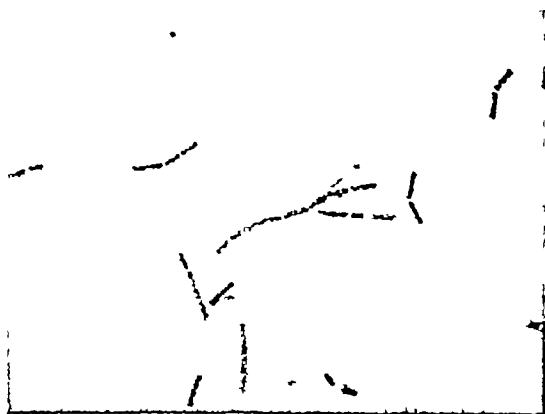


FIG 2

## Effect of Specific Antibody on the Capsule of Anthrax Bacilli

G BODON AND J TOMCSIK

*From the Department of Hygiene, Francis Joseph University, Szeged, Hungary*

The characteristic effect exerted by the type specific immune serum on the capsule of pneumococci, a phenomenon observed by Neufeld, induced several investigators (Sabin,<sup>1</sup> Armstrong<sup>2</sup> and Logan and Smeall<sup>3</sup>) to work out a new rapid method for typing pneumococci. Apart from its practical importance this reaction is of theoretical interest, since it contributes direct proof to the previous supposition that the antipneumococcus immune sera induce in a specific way the alteration of the bacterial capsule. Tulczynska<sup>4</sup> demonstrated that this phenomenon occurs also in case of other encapsulated bacteria, pneumobacillus and streptococcus. Similar experiments performed by her with anthrax bacilli gave however very inconclusive results.

It seems somewhat striking that whereas studies on anthrax contributed perhaps most to the elucidation of the basic facts of immunity, and that the relation of capsule formation to bacterial virulence was first demonstrated with this microorganism, yet our present knowledge on the effect of immune serum on the capsule of the anthrax bacilli is considerably less than in many other bacteria.

Our previous work<sup>5</sup> revealed the existence of an antibody produced by immunization with encapsulated anthrax bacilli and probably acting on the bacterial capsule.

The strain used for the major part of our study produced abundant capsule on agar media. Its 24 hours' growth was very moist and sticky and it killed rabbits with typical symptoms of anthrax infection when a 1/1000 part of a 24 hour agar slant culture was injected subcutaneously. The strain was isolated from the "Carbozoo" vaccine.<sup>7</sup>

Fig. 1 represents a wet India ink preparation showing that almost all of the bacilli are surrounded by well developed capsules.

<sup>1</sup> Sabin, *J. Inf. Dis.*, 1930, **16**, 469.

<sup>2</sup> Armstrong, R. R., *Brit. Med. J.*, 1931, **1**, 214, 1932, **1**, 137.

<sup>3</sup> Logan and Smeall, *Brit. Med. J.*, 1932, **1**, 188.

<sup>4</sup> Tulczynska, R. E., *Z. f. Hyg. u. Inf.*, 1933, **114**, 769.

<sup>5</sup> Tomcsik, J., and Szongott, H., *Z. f. Immunitätsforsch.*, 1933, **77**, 86.

<sup>6</sup> Tomcsik, J., and Bodon, G., *Proc. Soc. Exp. Biol. and Med.*, 1934, **32**, 118.

<sup>7</sup> Mazzucchi, M., *La Clinica Veterinaria*, 1931, **9**, 3.



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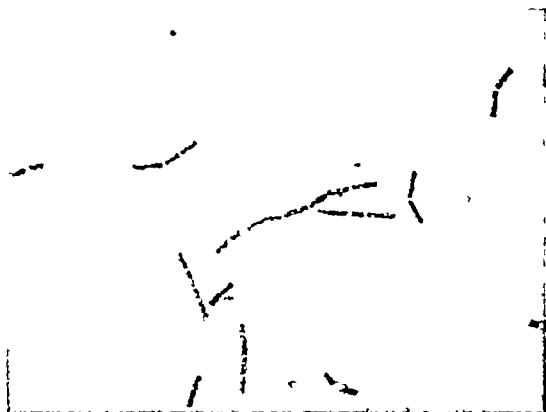


FIG 2

(2) those containing both antipolysaccharide and antiprotein precipitins. The effect of 10 different sera belonging to the first group had been studied with completely negative results, that is, the capsules did not turn visible after the addition of these sera (Fig 2).

An entirely different picture was observed when we employed 5 sera belonging to the second group. The addition of one loopful of these sera caused agglutination as reported previously. Apart from this the bacteria not included in the large clumps showed a characteristic change. The capsule showed up 1 or 2 minutes after the addition of the serum as a bright body with brown color and with a sharply defined outline toward the fluid (Fig 3).

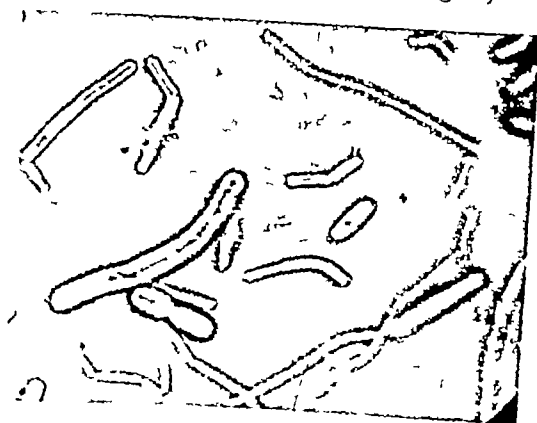


FIG 3

The method we adopted finally to show the effect of this immune serum on the capsule was the following. Two loopfuls of the 24 agar culture of the encapsulated bacilli were suspended in 0.5 cc salt solution. One loopful of this fairly homogeneous suspension was mixed on a cover-slip with the same quantity of Loeffler's methylene blue as well as of the immune serum containing P precipitins. The cover-slip containing this mixture was then inverted and placed over a hollow ground slide and sealed with vaseline. The capsule became visible as a rule after a few minutes owing to its changed refraction. In many instances it was stained as a pink body around the light blue bacilli due to the metachromatic effect of this stain.

In comparing the thickness and the appearance of the capsule with that in the India ink preparation, we had the impression that the visibility of the capsule was not due to a layer of immune serum surrounding and covering it, but rather to a specific reaction which

changed the refraction and the staining properties of the capsule proper

Altogether 12 different encapsulated and 9 other anthrax strains were examined in this way. We never failed to observe the closest parallelism in demonstrating the capsule production in India ink preparation and in our specific capsular reaction.

The correlation of the agglutinability of our strains and of the capsular reaction was also complete. The capsular reaction therefore has to be regarded as a visible sign of the union of anticapsular antibody with the capsular substance. The agglutination must be in consequence a secondary reaction following the specific alteration of the capsular material.

We believe that it would be of great importance to study in infected animals the protecting role of an antibody exhibiting such a marked specific action on the capsule of anthrax bacilli.

*Summary* Anthrax immune serum containing P precipitin exerts a specific effect on the capsule of anthrax bacilli. Following the addition of a small quantity of this immune serum to the suspension of encapsulated anthrax bacilli, the capsular material is specifically affected and becomes visible in unstained preparations. This is the final proof of the existence of a separate antibody in specially prepared immune serum acting in a specific way on the capsule of anthrax bacilli.

### 7583 C

#### Stability of Toxin Producing Attribute of Scarlet Fever Strains of Streptococci

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Several reports have appeared concerning the stability of the toxin producing power of scarlet fever strains of streptococci. Organisms dried on swabs or in cultures have been found to retain the toxin producing attribute (Jettmar,<sup>1</sup> Tunnichliff<sup>2</sup>). Tunnichliff<sup>3</sup> reported also that filtrates of certain different appearing colonies of

<sup>1</sup> Jettmar, M. H. v., *Z f Hyg u Infektionskr*, 1927, 107, 265

<sup>2</sup> Tunnichliff, R., *J Infect Dis*, 1927, 41, 272

<sup>3</sup> Tunnichliff, R., *J Infect Dis*, 1927, 48, 511

strains which had been cultivated for long periods of time were toxic as were filtrates of recently isolated strains when measured by the paramecium test. However, the filtrates of the dissociated strains were not neutralized by scarlet fever antitoxin. Pilot and Stocker<sup>4</sup> reported a non-hemolytic variant of a scarlet fever strain which was toxigenic. Friedemann and Deicher<sup>5</sup> passed 2 scarlet fever strains 10 times through mice and found that the filtrate of one strain had lost its toxigenic power while in the other strain the toxin producing power had increased. In the work here reported, comparative skin tests were made with filtrates of the original strains and filtrates of these strains after subjecting them to various methods used to dissociate bacteria.

From 3 to 9 strains were used in each method. The original strains had toxin titers ranging from 1:500 to 1:5000. All tests were done on 2 adults who gave consistent results with the various dilutions. Neither of these individuals reacted to injections of toxic filtrates from erysipelas and septic sore throat strains. Dissociation of the strains was attempted by

1. Subculturing daily in homologous immune rabbit serum diluted 1:10 in infusion broth. These sera were produced by the method used in preparing sera for the dissociation of pneumococcus. The sera contained agglutinins which clumped the homologous streptococcus strains at dilutions of 1:640 or greater. Nine strains were used and the number of transfers varied from 60 to 128.

2. Subculturing daily 4 strains in 1% glucose infusion broth for 60 to 121 transfers.

3. Subculturing daily 3 strains in infusion broth containing methylene blue at a concentration of 0.00001 mol. for 62 to 114 transfers.

4. Subculturing daily 4 strains in infusion broth and incubation at 45°C. for 60 to 119 transfers.

5. Passing 3 strains from 7 to 12 times through mice by intraperitoneal injections and recovery of the organisms from heart blood or peritoneal fluid.

6. From 1 strain 5 atypical colonies were selected which were apparently similar to some of the various dissociated colony forms described by others. Determinations of electrophoretic velocities as well as tests of toxigenicity were made with these strains.

In each instance the toxins, produced by all strains treated by

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<sup>4</sup> Pilot, I., and Stocker, S., *Proc. Soc. Exp. Biol. and Med.*, 1933, **31**, 181.

<sup>5</sup> Friedemann, U., and Deicher, H., *Z. f. Hyg. u. Infektionskr.*, 1928, **108**, 192.

these 5 methods, gave reactions to the same dilutions as those of the filtrates of the original cultures

In the case of the 5 colonies selected from one culture, while all of these produced filtrates which gave reactions at the titer of the original cultures, 3 gave reactions smaller in extent than the other 2. Cataphoresis determinations made on 2 occasions showed that the rate of migration of the organisms producing the smaller reactions was about twice that of the other 2 and of that of original culture. It should be stated, however that we have previously shown<sup>6</sup> that a faster rate of migration is not a specific characteristic of non-toxin producing strains of streptococcus.

*Summary* The use of several recognized methods for dissociation of bacteria when applied to scarlet fever strains of streptococci failed to deprive these strains of their ability to produce toxin.

## 7584 C

### On the Motion of Growth IX A Scheme for Analysis of Experiments on Growth, Nutrition and Metabolism.

NORMAN C WETZEL

*From the Babies and Childrens Hospital, Cleveland, and the Department of Pediatrics, School of Medicine, Western Reserve University*

The quantitative relationships between normal growth and heat production which the author has recently found and applied in the case of bacterial cultures<sup>1</sup> in *Bufo vulgaris* from fertilization throughout metamorphosis<sup>2</sup> and from birth to adult life in man<sup>3</sup> should likewise be helpful in dealing with the results of many experimental studies on growth, nutrition, or metabolism.

Such studies are carried out, almost without exception, upon the young of some species, and noteworthy, in the present connection on subjects still immersed in the "flux of growth." It is just at this stage of life, moreover, that characteristic and often conspicuous changes in metabolism are known to occur. Sufficient evidence in the 3 normal cases we have mentioned has already been brought forward to show that these changes in metabolism, as portrayed in

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<sup>6</sup> Thompson, R. L., and Megrail, E., *Am J Hyg*, 1934, 19, 457.

<sup>1</sup> Wetzel, N. C., *Proc Soc Exp Biol and Med*, 1932, 30, 360.

<sup>2</sup> Wetzel, N. C., *Proc Nat Acad Sc*, 1934, 20, 183.

<sup>3</sup> Wetzel, N. C., *Proc Soc Exp Biol and Med*, 1932, 30, 227, 233, *J Pediat*, 1933, 3, 252, 1934, 4, 465.

the data of other workers, are actually due to, and depend uniquely upon, the underlying and concurrent changes in growth itself

Thus, taking the relation between *growth*,  $q$ , and *size*,  $z$ , to be, as before,<sup>2, 4</sup>  $q = \mu \log_e \frac{z}{z_0}$ , with  $\mu = 1 = z_0$ , we may express the interdependence of growth and metabolism for the case of laboratory animals as follows

$$\left. \begin{array}{ll} \text{Growth} & \lambda \frac{d^2 q}{dt^2} + \rho \frac{dq}{dt} + \frac{q}{\kappa} = E \quad [\text{Cal} / \text{M} / \mu] \\ \text{Metabolism} & \rho \left( \frac{dq}{dt} \right)^2 + E_c \frac{dq}{dt} + A' = U \quad [\text{Cal} / \text{M} / \text{T}] \end{array} \right\} (1)$$

the significance and dimensions of individual symbols having been outlined and applied elsewhere.<sup>2, 4</sup> For present purposes, however, we have briefly  $q$ , the rate of *growth* as distinguished from the rate of *gain* (less commonly *loss*),  $z$ ,  $\rho$ , the resistance  $\lambda$ , the inductance,  $\kappa$ , the permittance of growth,  $E$ , the net external work of growth, and  $E_c$ , the work of synthesis, each of the 2 latter constants being referred to the unit of mass ( $z$ ) and charge of growth ( $q$ ), and finally,  $A'$ , the heat of maintenance, in terms of power per unit mass liberated even when growth is in the state of rest, that is, when  $\ddot{q} = \dot{q} = 0$

These results lead to several suggestions for further experimental work in the fields of growth, nutrition, and metabolism. For the methods which have succeeded in establishing the dynamic connection (1) between the concomitant events of pure growth on the one hand, and those of heat production or metabolism on the other, would now appear to be of considerable assistance in the analysis of various questions arising in these fields when growth itself is directly or indirectly put to experimental test

How, for example, does a small quantity of lettuce incorporated into the usual diet of young white rats lead to an increase over the "normal" rate of gain?<sup>5</sup> How, also, does the administration of anterior pituitary extract produce a somewhat similar result, the treated rats ultimately weighing about 16% more than the controls,<sup>6</sup> notwithstanding the fact that the quantity of food remained the same in each group, or, even more unexpectedly, in spite of the fact that the fuel value of sacrificed carcasses proved to be less in the test animals than in the controls?<sup>7</sup> The former, it was found, contained less fat. Their tissues possessed, on the whole, the chemical

<sup>4</sup> Wetzel, N. C., *Proc. Soc. Exp. Biol. and Med.*, 1933, **30**, 1044.

<sup>5</sup> Outhouse, Julia, and Mendel, L. B., *J. Exp. Zool.*, 1933, **64**, 257

<sup>6</sup> Lee, M. O., and Schaffer, N. K., *J. Nutrition*, 1934, **7**, 337



constituents recognized as characteristic of younger normal animals, and the changes in body composition usually associated with advancing age, increasing size, and with progress toward maturity, did not take place

The foregoing combination of results is one of the various possible groups of effects to be expected when growth is artificially distorted, but it also turns out that precisely the combination which Lee and Schaffer describe should likewise be accompanied by decreased heat production. This is of especial interest since these authors have been able to show that the administration of anterior pituitary growth hormone is followed by a drop in metabolism.<sup>6</sup> How, then, have the hormone, food, and growth combined, while acting together, to yield such results?

Other questions are likewise suggested. What initiates the process of growth, or which factors are concerned with the natural acceleration of growth that usually takes place after the onset? What, finally, is responsible in chief part for the fact that growth in healthy structures is strictly confined within "normal" limits, and to what, therefore, is "disorganized" growth in unhealthy tissues to be ascribed?

Questions such as these are not necessarily to be considered as beyond the hope of further analysis or investigation. It should, in fact, be possible to attack them on the experimental side with the promise that definite answers might be obtained in terms of the fundamental parameters set out in equation (1). Let us see how the matter could be expected to work out in practice.

To fix ideas, we assume that an investigator proposes to study several substances or procedures, say  $x_1, x_2, \dots, x_n$  by means of their respective effects upon the young of certain species, whose domesticated "normal" rate of growth,  $(q)_0$ , may be written, in view of the first of equations (1), in the functional form,

$$(q)_0 = F[\rho_0, \lambda_0, \kappa_0, (E)_0, \dots, (q)_0, (\bar{q})_0] \quad (2)$$

$F$ , accordingly, being characteristic of the species. Now,  $(q)_0$  will be altered by any one, or suitable combinations of changes in the parameters,  $\rho_0, \lambda_0, \dots$  induced by some external agent, as here by  $x_1, x_2, \dots, x_n$ , and we shall have,

$$\left. \begin{aligned} (q)_1 &= F[\rho_1, \lambda_1, \kappa_1, (E)_1, \dots, (q)_1, (\bar{q})_1] \\ (q)_n &= F[\rho_n, \lambda_n, \kappa_n, (E)_n, \dots, (q)_n, (\bar{q})_n] \end{aligned} \right\} (3)$$

$F$  remaining the same since the species is unchanged

The problems occurring in practice are, however, just the converse of this, for the worker will wish to know which parameters are changed when the normal or control rate of growth,  $(q)_0$ , has been experimentally altered. Such a problem is clearly beyond solution, as may be inferred from (1) so long as experimental observations are limited to a study of change in size,  $z$ , alone. The best that could be done would be a determination of the ratio  $\rho/\lambda$ , which has the dimension  $[T^{-1}]$  simply. But, since  $q_{(1)}, \dots, q_{(n)}$  can be computed with the help of (1) and (2) from the experimental measurements of size, it is evident that a simultaneous study of heat production will at once permit the numerical evaluation of the individual constants,  $\rho_1, \rho_2, \dots, \rho_n, \lambda_1, \lambda_2, \dots, \lambda_n, (E_0)_1, (E_0)_2, \dots, (E_0)_n$ , etc., since the data on heat may be adjusted to the accompanying equations,

$$\left. \begin{aligned} (U)_1 &= \phi[\rho_1, (E_0)_1, (A')_1, (q)_1, (q)_1^*] \\ (U)_n &= \phi[\rho_n, (E_0)_n, (A')_n, (q)_n, (q)_n^*] \end{aligned} \right\} (4)$$

for substances  $v_1, \dots, v_n$  respectively, the function  $\phi$  remaining the same as long as the observations are taken on the same species of organism. Equations (4), accordingly, provide a method of determining the  $\rho$ 's and hence, with the latter known, a method for computing all of the remaining constants along with their probable errors if least square procedure be adopted as in the case of *Bufo vulgaris* previously reported.<sup>2</sup> In experiments  $v_1, v_2, \dots, v_n$  we shall obviously meet the various possibilities suggested by the relations

$$\left. \begin{aligned} \rho_1 &\left. \begin{array}{l} > \\ = \\ < \end{array} \right\} \rho_0, & \lambda_1 &\left. \begin{array}{l} > \\ = \\ < \end{array} \right\} \lambda_0, & (E_0)_1 &\left. \begin{array}{l} > \\ = \\ < \end{array} \right\} (E_0)_0 \end{aligned} \right\} (5)$$

The effect, therefore of any foodstuff, or of any procedure that influences growth may always be estimated in terms of the fundamental properties of growth,  $\rho, \lambda, \kappa$ , and  $E$ , which are held to govern the motion of growth in that particular system. Whether such substances "promote" or "inhibit" growth, their action will become manifest by means of the respective changes which they induce in the normal control values of these 4 parameters of state. The point should ultimately be reached where the more important substances or procedures  $x_1, x_2, \dots, x_n$  (e.g., vitamins, hormones or other materials) could be classified with respect to their several or indi-

vidual effects upon the fundamental properties of growth,  $\rho$ ,  $\lambda$ ,  $\kappa$ ,  $E$ . At that stage it will have become apparent whether a suitable choice of the  $r$ 's will permit growth to be placed under full control or not.

*Summary* (1) Post-embryonic growth of common laboratory animals is governed, in accordance with the first of equations (1), by 4 fundamental properties of growth represented by the constants  $\rho$ ,  $\lambda$ ,  $\kappa$ , and  $E$ . (2) Rates of growth ( $q$ ) are altered when any one, or suitable combinations of these parameters are changed by experimental means. In practice, however, the problem is more likely to be the converse of this: which parameters are changed when the normal or control rate of growth is known to have been altered? Such a problem is insoluble so long as observations are limited to measurements of change in size,  $z$ , alone. (3) Heat production "during growth" ( $q \neq 0 \neq q$ ) is quantitatively different from heat production when growth is in the stationary state ( $\bar{q} = q = 0$ ). Heat production per unit time per unit mass is synonymous with metabolism, and the latter is dynamically related to growth *via* the properties represented by  $\rho$  and  $E$ . (4) The values of all constants along with their  $PE$ 's can be computed from simultaneous data on growth and metabolism. (5) The effect of any foodstuff, or of any procedure that influences growth can therefore be estimated in terms of the control values  $\rho_0$ ,  $\lambda_0$ ,  $\kappa_0$ , and  $(E)_0$ , and the substances themselves may be compared by means of the respective changes induced in these four fundamental parameters of state.

## 7585 C

### Effect of Certain Physical Factors on the In Vitro Testing of Anthelmintics \*

PAUL D. HARWOOD (Introduced by P. D. Lamson.)

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Although certain investigators have done much to destroy confidence in the value of *in vitro* methods of testing anthelmintics by drawing too sweeping conclusions from uncontrolled experiments, these methods are of value and were used successfully by Lamson

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\*The funds for carrying out this work were given by the International Health Division of the Rockefeller Foundation.

*et al*<sup>1</sup> in their studies on the alkyl resorcinols. These studies have resulted in the establishment of hexyl resorcinol as an effective ascaricide of relatively low toxicity. This method of *in vitro* testing of ascaricides is described elsewhere,<sup>2</sup> but there remains for discussion certain physical factors which must be guarded against if reliable results are to be obtained.

As Lo Monaco<sup>3</sup> has already shown with santonin, an undissolved excess of the drug must be present in the testing solution for the best results, but he makes no mention of the effect of the physical state of the excess. However, a solid excess is far less effective than a liquid excess as will be shown. If certain substances, such as heptyl resorcinol, are allowed to stand for sometime in contact with 1,000 parts of 0.9% NaCl solution at 37° C, a part of the chemical will be dissolved, but as these substances are less soluble than 1 to 1,000 a solid excess will remain. If another sample of this same chemical is heated in contact with the same relative amount of 0.9% NaCl and cooled to 37° C we shall have 2 mixtures of the drug and saline with the sole difference that in one instance the undissolved excess is a solid, while in the other it is a supercooled liquid. When these 2 mixtures are tested against *Ascaris lumbricoides* of swine, it is found that the one with liquid excess will kill the worms in a much shorter time. Table I records the results obtained when several of these substances were tested with both a solid and a liquid excess.

But many solids which have possible uses as anthelmintics will not remain liquid when cooled to 37°. It has been necessary to adulterate these higher melting substances with some organic liquid to keep them in the liquid phase. Many liquids have been tried in this laboratory with that purpose in mind, but usually the dilutant was either toxic to the worms or it inhibited to some degree the activity of the drugs being tested. n-Hexane has served this purpose better than any other chemical as it is non-toxic to the worms and has comparatively little effect upon the drug to be tested. Table II shows the results obtained when solid substances are liquefied with n-hexane.

It is well known that the molecules of a substance in a liquid state are much more motile than molecules of the same substance in a solid state. In the former case they are in no particular relation to one another but may freely move about in the liquid mass,

<sup>1</sup> Lamson, P. D., Brown, H. W., Ward, C. B., and Robbins, B. H., *Proc. Soc. Exp. Biol. and Med.*, 1930, 28, 191.

<sup>2</sup> Lamson, P. D., Brown, H. W., and Harwood, P. D. In Press.

<sup>3</sup> Lo Monaco, *Arch. ital. de Biol.*, 1896, 26, 216.

TABLE I

Drug	Melting point	Exposure necessary to kill <i>A. lumbricoides</i> in	
		solid excess	liquid excess
1 propyl naphthol 2	57°	min. 10	min. 2 5
2 propyl naphthol 1	49°	30 45	10 20
amyl resorcinol	71 5 73°	15	2
heptyl "	73 74.5°	5 10	2
o phenyl phenol	56°	20 60	5

TABLE II

Drug	Melting point	Amount of n hexane	Exposure necessary to kill <i>A. lumbricoides</i> with	
			pure drug	adulterated drug
p (3 amyl) phenol	75 5-76°	% 1/8	min 20	min. 2
cyclo hexyl resorcinol	128°	1	180	2
p tertiary amyl phenol	93 94°	1	20	2
p tertiary butyl "	97 98°	1	20	2
p chlorothymol	64°	1/8	20	5

while molecules in a solid have certain more or less definite positions relative to their neighbors and are accordingly, more restricted in their movements. A liquid will, therefore, dissolve much more rapidly in another liquid than the same substance in a solid state would dissolve in the same solvent. With these slightly soluble anthelmintics the removal from the test solution of a small amount of the drug would appreciably lower the degree of saturation. With a well agitated mixture this situation would be rapidly remedied if a liquid excess of the drug is present. However, a solid excess with its less motile molecules would be much slower in replacing the removed drug, and the test animals would be exposed to a much lower concentration of the drug. Furthermore, a liquid excess would be able much more readily to penetrate directly into the worm without passing through a dissolved phase than would be the case with a solid excess.

## Effect of a High Salt Diet on Survival of Adrenalectomized Rats

ROBERT GAUNT, CHARLES E TOBIN, AND JO HOWLAND GAUNT

(Introduced by W W Swingle)

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That NaCl is beneficial in the treatment of experimental adrenal insufficiency was established by the earlier work of Stewart and Rogoff,<sup>1</sup> Banting and Gairns,<sup>2</sup> Marine and Baumann,<sup>3</sup> and Corey,<sup>4</sup> and the recent work of Loeb *et al.*,<sup>5</sup> Harrop *et al.*,<sup>7</sup> Swingle *et al.*,<sup>8</sup> Zwemer,<sup>9</sup> and Rubin and Krick.<sup>10</sup>

A consensus of the findings is that NaCl feeding in dogs and cats will prolong but not indefinitely maintain, life after total adrenal ablation. Rubin and Krick found, however, that in 8 rats a drinking solution of 0.0329% CaCl<sub>2</sub>, 0.015% MgCl, 0.07% NaCl and 0.035% KCl given upon the appearance of adrenal insufficiency symptoms, would maintain life for 4 months or more in animals which normally would not live longer than 10 days. At the time of their publication these authors had apparently not determined, by discontinuing treatment, whether accessory adrenals had assumed a functional condition. From their work it would appear that the rat, unlike the cat and dog, will live indefinitely if fed a high salt diet after adrenalectomy. At the time of Rubin and Krick's publication we were studying the effects of adding salt to the diets of adrenalectomized rats. In addition to this we adopted their technique of adding salt to the drinking water, a method probably more effective.

In previous experience with our rat colony we found that ap-

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<sup>1</sup> Rogoff, J M, and Stewart, G N, *Am J Phys*, 1928, **84**, 649

<sup>2</sup> Banting, F G, and Gairns, S, *Am J Phys*, 1926, **77**, 100

<sup>3</sup> Marine, D, and Baumann, E J, *Am J Phys*, 1927, **81**, 86

<sup>4</sup> Corey, E L, *Am J Phys*, 1927, **70**, 633

<sup>5</sup> Loeb, R F, *Proc. Soc. Exp. Biol. and Med.*, 1933, **30**, 808

<sup>6</sup> Loeb, R F, Atchley, D W, Benedict, E M, and Leland, J J, *J. Exp. Med.*, 1933, **57**, 775

<sup>7</sup> Harrop, G A, Soffer, L J, Ellsworth, R, and Trescher, J H, *J. Exp. Med.*, 1933, **58**, 17

<sup>8</sup> Swingle, W W, Pfaffner, J J, Vars, H. M, and Parkins, W M, *Am J Phys.*, 1934, **108**, 159

<sup>9</sup> Zwemer, R L, *Endocrinology*, 1934, **18**, 161

<sup>10</sup> Rubin, M. L, and Krick, E T, *Proc. Soc. Exp. Biol. and Med.*, 1933, **31**, 228

proximately 95% do not survive adrenalectomy<sup>11</sup> In the latest adult control series 4 out of 24 survived longer than 30 days, 2 longer than 50 days

In the present experiments, 1.5% NaCl was added to the stock diet\* in part of the cases, 2.5% in the others Either the Rubin-Krick salt solution or 0.9% NaCl solution was given the animals to drink As far as we could tell one of these feeding-drinking combinations was no more effective than another, so further distinctions between them are not made here

Twenty-three young, but mature, adrenalectomized rats, weighing from 140 to 200 gm, were studied The salt treatment was continued for 30 days after adrenalectomy, at which time distilled water and the stock diet were substituted The results of this treatment from the standpoint of survival can be conveniently divided into 3 categories

1 Five cases, unlike those of Rubin and Krick, succumbed from 14 to 25 days after operation, *i e*, during the course of treatment with typical symptoms of adrenal insufficiency

2 Six animals survived and gained weight during the course of the treatment, were apparently in good condition when the treatment was stopped, but after being returned to normal diet developed adrenal insufficiency and died in 10 to 15 days

3 Twelve animals, approximately 50%, survived in good condition while treated, and after treatment was discontinued gained weight and remained in apparently normal health until killed for autopsy 8 or more weeks after operation In 7 of these animals accessory adrenals were found

The survival of 50% after treatment was discontinued is similar to the results we obtained in this colony after withdrawing cortical hormone treatment<sup>12</sup> Thus it would appear that any agent that will delay the appearance of adrenal insufficiency will, probably by allowing time for the hypertrophy of accessories, cause indefinite survival in this colony in about 50% of the cases †

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<sup>11</sup> Gaunt, R., *Am J Phys*, 1933, 103, 494

\*Our stock diet is composed of 9 parts by weight of GLF Calf Meal, and one part ground meat and bone scrap To this is added yeast, cod liver oil, and lettuce.

<sup>12</sup> Gaunt, R., and Gaunt, J. H., *Proc Soc Exp Biol and Med*, 1934, 31, 490

†Since these experiments were completed we have carried out a series of similar studies using animals operated at 30 days of age This latter work is sufficiently complete to indicate that the results obtained are similar to those reported above for adults, although the total fatalities are considerably greater

Of 46 untreated controls only 2 (4.3%) survived for as long as 2 months and

## Effect of a High Salt Diet on Survival of Adrenalectomized Rats.

ROBERT GAUNT, CHARLES E TOBIN, AND JO HOWLAND GAUNT  
(Introduced by W W Swingle)

From the Department of Biology, College of Charleston, S C, and the Biological Laboratory, Cold Spring Harbor, L I

That NaCl is beneficial in the treatment of experimental adrenal insufficiency was established by the earlier work of Stewart and Rogoff,<sup>1</sup> Banting and Gairns,<sup>2</sup> Marine and Baumann,<sup>3</sup> and Corey,<sup>4</sup> and the recent work of Loeb *et al.*,<sup>5, 6</sup> Harrop *et al.*,<sup>7</sup> Swingle *et al.*,<sup>8</sup> Zwemer,<sup>9</sup> and Rubin and Krick.<sup>10</sup>

A consensus of the findings is that NaCl feeding in dogs and cats will prolong but not indefinitely maintain, life after total adrenal ablation. Rubin and Krick found, however, that in 8 rats a drinking solution of 0.0329% CaCl<sub>2</sub>, 0.015% MgCl, 0.07% NaCl and 0.035% KCl given upon the appearance of adrenal insufficiency symptoms, would maintain life for 4 months or more in animals which normally would not live longer than 10 days. At the time of their publication these authors had apparently not determined, by discontinuing treatment, whether accessory adrenals had assumed a functional condition. From their work it would appear that the rat, unlike the cat and dog, will live indefinitely if fed a high salt diet after adrenalectomy. At the time of Rubin and Krick's publication we were studying the effects of adding salt to the diets of adrenalectomized rats. In addition to this we adopted their technique of adding salt to the drinking water, a method probably more effective.

In previous experience with our rat colony we found that ap-

<sup>1</sup> Rogoff, J M, and Stewart, G N, *Am J Phys*, 1928, **84**, 649

<sup>2</sup> Banting, F G, and Gairns, S, *Am J Phys*, 1926, **77**, 100

<sup>3</sup> Marine, D., and Baumann, E J, *Am J Phys*, 1927, **81**, 86

<sup>4</sup> Corey, E L, *Am J Phys*, 1927, **79**, 633

<sup>5</sup> Loeb, R. F, *Proc. Soc. Exp. Biol. and Med.*, 1933, **30**, 808

<sup>6</sup> Loeb, R. F, Atchley, D W, Benedict, E M, and Leland, J J, *J Exp Med.*, 1933, **57**, 775

<sup>7</sup> Harrop, G A, Soffer, L. J, Ellsworth, R, and Trescher, J H, *J Exp Med.*, 1933, **58**, 17

<sup>8</sup> Swingle, W W, Piffner, J J, Vars, H M, and Parkins, W M, *Am J Phys*, 1934, **108**, 159

<sup>9</sup> Zwemer, R L, *Endocrinology*, 1934, **18**, 161

<sup>10</sup> Rubin, M. I, and Krick, E T, *Proc. Soc. Exp. Biol. and Med.*, 1933, **31**, 228



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<sup>12</sup> Gaunt, R., and Gaunt, J. H., *Proc Soc Exp Biol. and Med*, 1934, **31**, 490

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Of 46 untreated controls only 2 (4.4%) survived for as long as 2 months and

Twelve out of 14 attempts to revive animals in the late stages of adrenal insufficiency, either by intraperitoneal normal saline injections or by feeding normal saline or the Rubin-Krick salt solution were unsuccessful. In 2 cases revival was effected. These revivals were not attempted until a fall in body temperature indicated severe adrenal insufficiency, although the animals could in all probability in every case have been revived with cortical extract.

7587 P

### Improved Colorimetric Method for Determination of Bromide Concentration in Blood and Cerebrospinal Fluid

S KATZENELBOGEN AND T CZARSKI.

*From the Phipps Psychiatric Clinic, Johns Hopkins Hospital*

In our previous study<sup>1</sup> dealing with determinations of bromide in blood and in cerebrospinal fluid it was pointed out that with the colorimetric procedure of Hauptmann<sup>2</sup> one cannot recover the actual amount of bromide in blood serum. According to Wuth's assumption the precipitates of blood proteins retain a certain amount of bromide.<sup>3</sup> However, with another method<sup>4</sup> for the bromide determination in which, like Wuth, we also used protein-free filtrates, we were always able to recover the total amount of bromide dissolved in various specimens of blood serum.

Having inferred from these findings that proteins are not essentially responsible for the loss of bromide, we tried out *in vitro* the effect of various blood components on the bromide determination. Dissolving, respectively, uric acid, creatinine, urea, glucose, amino acids (glycine), lactic acid, potassium sulfate, sodium carbonate, magnesium-ammonium phosphate, potassium iodide and sodium

these when killed for autopsy showed large accessories. Forty four (95.6%) died within 34 days, the average survival was 8 days.

In 41 animals given salt treatment for 30 days after operation, only 18 (43.9%) died during the course of treatment, the average survival being 9 days. Twenty three animals (56.1%) were alive when the treatment was discontinued. At this writing the survival of this group, after treatment was stopped, has not been determined.

<sup>1</sup> Katzenelbogen, S, and Goldsmith, H, *Am J Psych*, 1931, 10, 1045

<sup>2</sup> Hauptmann, A., *Klin Wochenschr*, 1925, 4, 1629

<sup>3</sup> Wuth, O, *J A M A*, 1927, 88, 2013

<sup>4</sup> Hastings, A. B, and van Dyke, H. B, *J Biol Chem*, 1931, 92, 24.

chloride in aqueous solutions of sodium bromide, we found that only sodium chloride influenced the results obtained with Hauptmann's colorimetric determination of bromide. The more sodium chloride added, the lighter became the color, and the less bromide was found.

On the basis of this observation we attempted to modify Hauptmann's procedure, so as to eliminate the error arising from the presence of sodium chloride. The principle of our modification consists in equalizing (as nearly as possible) the NaCl concentration in the blood serum, spinal fluid and standard before carrying out the color reaction (due to formation of gold bromide). For that purpose we used, instead of distilled water, salt solutions for the dilution of the blood serum and for the preparation of the standard. In view of the fact that the cerebrospinal fluid commonly contains considerably less bromide than the blood serum, it is used undiluted for the bromide analysis. No sodium chloride need be added to the cerebrospinal fluid because its sodium chloride concentration very nearly approaches that of our blood serum dilutions and of the standards prepared with salt solutions.

TABLE I.

Experiments with serum			Experiments with spinal fluid		
NaBr added	(1) NaBr recovered	(2) NaBr recovered	NaBr added	(1) NaBr recovered	(2) NaBr recovered
50	42.0 (-16.0)	50.5 (+1.0)	10	8.6 (-14.0)	10.2 (+2.0)
100	84.3 (-15.7)	100.0 (0)	20	17.2 (-14.0)	20.3 (+1.5)
150	126.7 (-15.7)	149.0 (-0.7)	40	33.3 (-16.7)	40.0 (0)
200	165.7 (-17.1)	197.5 (-1.3)	60	48.3 (-19.5)	58.3 (-2.8)
250	203 (-17.2)	246 (-1.6)	80	60.0 (-25.0)	80.7 (+0.9)
300	245 (-18.3)	297 (-1.0)	100	69.0 (-31.0)	103.5 (+3.5)

(1) by Hauptmann's procedure, (2) by the modified procedure

Figures are in mg % Figures in ( ) are differences in %

Table 1 shows that with the original Hauptmann's procedure losses of bromide were registered, conversely, with our modified procedure the amounts of sodium bromide dissolved in various specimens of blood serum and of cerebrospinal fluid were totally recovered.

The technique of the modified method is essentially the same as the technique previously described,<sup>1</sup> except for the following differences: (a) Dilution of blood serum with a 0.75% NaCl solution (instead of water). (b) The stock solution contains NaBr 166.8 mg % (instead of 142 mg %) in a 0.70% NaCl solution (instead of water). (c) The working standards are prepared with a 0.70% NaCl solution (instead of water).

# Acid Fuchsin for Demonstration of Ingestion in *Paramecium caudatum*

ELBERT C COLE (Introduced by A. E. Adams)

*From the Department of Biology, Williams College*

The study of ingestion in *Paramecium* is ordinarily accompanied by considerable difficulty due to the fact that there is little difference in color between the animal, the surrounding medium, and the bacteria which serve as food. The following method differentiates these elements by means of contrasting colors.

The dye used in this investigation was acid fuchsin, certification number NR-2, having a dye content of 62%. Solutions were prepared with distilled water so that their described percentages represented the actual dye strength.

Two drops of culture fluid containing large numbers of paramecia were placed on a clean slide, a drop of 1% solution of acid fuchsin added, and a cover glass applied. The fluid formed a thick layer under the cover glass, but the dye solution was unequally distributed. The preparation was allowed to stand undisturbed for one or 2 minutes, then examined under low magnification with reduced light. The paramecia, under these conditions, appeared to be outlined sharply in black. A portion of the fluid was then drawn off by the application of a strip of filter paper, thus reducing the thickness of the film of solution under the cover glass. In this thinner layer paramecia appeared to be colored a pale, luminous green, the color being deeper in those animals which had spent some time in the more deeply colored portions of the preparation before swimming out into the lighter areas. It must be emphasized here that this color phenomenon appears only when the amount of liquid under the cover glass is small, it will not appear if the cover glass "floats high."

If a quiet *Paramecium* is studied under high magnification, the process of ingestion and the formation of food vacuoles may be seen with a diagrammatic clearness. The green animal lies in a red or pink environment. Bacteria, stained pink or red, are swept into the cytopharynx which, due to the color of its fluid contents, appears a delicate pink. The undulating membrane can be clearly seen in action. At the tip of the cytopharynx the forming food vacuole may be observed, its contents in rapid motion due to the continuous entry of additional water and food. The pink vacuole is sharply set

off from the surrounding green protoplasm. Finally the sharp contraction of the protoplasm at the tip of the cytopharynx may be observed, and the course of the newly formed food vacuole, with its pink and red contents followed in its passage through the cytoplasm.

It should be pointed out that this color phenomenon is in no way due to changes in pH. It is not, therefore, an indicator effect such as was described by Nirenstein.<sup>1</sup> The effect has been demonstrated in several microscopic organisms by the use of acid fuchsin, phenosafranin or erythrosin. It appears to be a physical phenomenon, closely related to the existence of extremely thin films of dye solution. Further study of this effect is under way.

## 7589 C

### Polarization Studies in Tissue Models \*

M SPIEGEL-ADOLF AND E. A. SPIEGEL

*From the Department of Colloid Chemistry and the Department of Experimental Neurology, D. J. McCarthy Foundation, Temple University School of Medicine, Philadelphia*

It is known that the electric resistance of animal tissues, contrary to that of solutions, is not determined by the law of Ohm alone.<sup>1</sup> This has been explained as consequence of polarization caused by semipermeable cell membranes at cell interfaces. The degree of polarization and, therefore, of the permeability can be measured by the capacitance or inductance needed to obtain a sharp minimum on the Wheatstone bridge. As further manifestation of polarization, the resistance of tissues decreases with the increasing frequency of the alternating current. While other authors worked mostly with methods based on the first phenomena, we used the difference in conductivity at high and low frequencies as measure of membrane polarization and permeability. In order to elucidate to some degree the chemical and colloid-chemical conditions underlying the polarization and permeability phenomena, different artificial membranes with various constituents were used.

A Wheatstone bridge was employed. Alternating currents of

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<sup>1</sup> Nirenstein, Edmund, *Z. f. wiss. Zool.*, 1925, **123**, 513.

\* Aided by a grant from the Ella Sachs Plotz Foundation.

<sup>1</sup> Gildemeister, M., *Handb. d. norm. und pathol. Physiol.*, 1928, **8**, 657.

various frequencies (560 to 6890 cycles) were provided by an oscillator built according to the description of Jones and Joseph,<sup>2</sup> in combination with an amplifier † The difference between the conductance at highest and lowest frequency was expressed in percentage of the conductivity at low frequency This value will be called  $\Delta$  in this paper In order to obtain a sharp minimum, variable capacities (up to 0.5 M F) were employed The conductance vessel consisted of 3 glass cells, the outer cells contained electrodes of platinum wire gauze covered with black of platinum Their surface was 3.8 cm<sup>2</sup>, the distance was 21 mm Between the outer cells and the middle cell one or 2 membranes could be placed The apparatus was filled with KCl solutions, whose concentration varied depending on the resistance of the membrane (0.1-1 n solutions) The method was first checked on frog's skins which gave a  $\Delta$  of 23% (high frequency 6890 cycles, low frequency 560 cycles)

**Results** When parchment, collodium, 30% gelatine membranes hardened in formaldehyde (Collander<sup>3</sup>), 30% gelatine membranes containing 2.5% pseudoglobulin were measured,  $\Delta$  did not exceed 0.4% Thus proteins alone seem to play no or only a minimal part in the mechanism of polarization observed in tissues

The behavior of lipid membranes (containing egg lecithin Merck, pure lecithin, or kephalin from human brains) depends on their preparation The first group of these membranes was prepared from collodium-ether solutions containing from 2-5% of the respective lipoids in molecular or lowly polymerized dispersion These membranes showed definite polarization phenomena (See Table I)

In the preparation of the second group of the lipid membranes, gelatine, up to a concentration of 30%, was added to watery colloid solutions of lecithin or kephalin While the lecithin-gelatin membranes were translucent, the kephalin-gelatin membranes were

TABLE I.

Lecithin in %	Frequency of the alternating current		$\Delta$ for 1 membrane	$\Delta$ for 2 membranes
	low	high		
2	1115	4860	4.7%	
2.5	560	4860	9%	40.6%
5	560	6890		71.2%
5	560	4860	15.7%	
5	560	6890	16.5%	

<sup>2</sup> Jones, G., and Joseph, R. C., *J Am Chem Soc*, 1928, 50, 1049

† We wish to express our thanks to Dr. G. Henny for his help in building the oscillator

<sup>3</sup> Collander, R., *Protoplasma*, 1927, 3, 213

opaque Nevertheless they gave the same results in the conductance measurements, both showing low values of  $\Delta$  (maximum 4% )

These experiments show that polarization phenomena as observed in animal tissues can be imitated by lipoids They show, furthermore, that the polarization, as measured by  $\Delta$ , depends on the degree of dispersion of the lipid in the membrane Membranes containing the lipid in fine dispersion show high degree of polarization (high value of  $\Delta$ ) as do animal tissues, while membranes with lipoids in coarse dispersion show no or only minimal polarization

This conclusion is corroborated by microscopical studies of the lipid membranes The collodium-*lecithin* membranes are homogeneous, while the *gelatin-lecithin* membranes show doubly refracting *lecithin* lumps in irregular distribution

The polarization of the lipid membranes (as expressed by  $\Delta$ ) was increased when the membranes were placed in 0.5 N HCl for 24 hours It was diminished or destroyed by analogous treatment with 0.5 N NaOH or 95% alcohol The change in the alcohol was irreversible due to the extraction of *lecithin* The polarization of *lecithin* membranes treated with alkali could be restored by subsequent exposure to acid ( 0.5 N HCl )

These experiments on membranes were followed by similar studies on brain tissue According to Nernst<sup>4</sup> and others, excitation is supposed to be due to a change of ion concentration on semi-permeable membranes Thus it seemed of interest to study agents that influence the convulsant reactivity in regard to their effect upon the polarization of brain tissue with this method It was found on the cerebral hemispheres of cats and guinea pigs that  $\Delta$  diminishes under the influence of agents that produce a swelling of the tissue (hypotonic salt solutions, alkali) These studies are being continued with special reference to convulsive reactions

## 7590 P

### Cause of Laxative Effect of Feeding Bran Pentosan and Cellulose to Man

WM. H. OLMSTED, GEORGE CURTIS AND O. K. TIMM

*From Washington University School of Medicine, St. Louis*

Recent work suggests that the laxative effect of bran in animals and man is due to its fibre content But in addition to fibre there

<sup>4</sup> Nernst, W., *Pflügers Arch. f. Physiol.*, 1908, 122, 275

various frequencies (560 to 6890 cycles) were provided by an oscillator built according to the description of Jones and Joseph,<sup>2</sup> in combination with an amplifier † The difference between the conductance at highest and lowest frequency was expressed in percentage of the conductivity at low frequency This value will be called  $\Delta$  in this paper In order to obtain a sharp minimum, variable capacities (up to 0.5 M F) were employed The conductance vessel consisted of 3 glass cells, the outer cells contained electrodes of platinum wire gauze covered with black of platinum Their surface was 3.8 cm<sup>2</sup>, the distance was 21 mm Between the outer cells and the middle cell one or 2 membranes could be placed The apparatus was filled with KCl solutions, whose concentration varied depending on the resistance of the membrane (0.1-1 n solutions) The method was first checked on frog's skins which gave a  $\Delta$  of 23% (high frequency 6890 cycles, low frequency 560 cycles)

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<sup>3</sup> Collander, R., *Protoplasma*, 1927, 3, 213



## 7591 C

## Effect of Gelatine Feeding upon Cases of Pseudohypertrophic Progressive Muscular Dystrophy \*

PAUL KISNER, EDWARD S WEST AND J ALBERT KEY

*From the Department of Biological Chemistry and the Department of Orthopedic Surgery, Washington University School of Medicine*

The beneficial effects of glycine feeding upon several cases of muscular dystrophy reported by Milhorat, Techner and Thomas<sup>1</sup> prompted the writers to investigate the effect of prolonged gelatine feeding upon 3 boys,<sup>2</sup> well advanced cases of pseudohypertrophic progressive muscular dystrophy, patients in the Shriners' Hospital for Crippled Children.† The boys, C E, M S, and D G were 11, 9 and 8 years of age, respectively. D G was able to walk but could not arise from a sitting position, C E and M S were unable to walk and could move only by sitting and pushing themselves along with their hands. The boys were removed from bed each morning and encouraged to exercise as much as they would during the day. The period of study extended throughout most of one year and included a preliminary control period on a meat-free diet, a prolonged period of gelatine feeding without meat, followed by a period without gelatine. Creatinine and creatine determinations were run on carefully collected daily urine samples. At intervals the ability of the 3 children to exercise was determined by having them walk (or slide if unable to walk) until exhausted, a record being kept of the distance covered. A tabulation of data on C E, the most severe case, is given in Table 1 as illustrative of the findings in all these cases. Gelatine feeding markedly increased the excretion of creatine in all cases as previously found by Gibson and Martin.<sup>3</sup> The increase was greatest in C E, the most severe case, and least in D G, the mildest case. An increase followed by a decrease was observed as found by Milhorat, Techner and Thomas during glycine

\*The writers are indebted to the Charles B Knox Gelatine Company, who kindly supplied the gelatine for the investigation.

<sup>1</sup> Milhorat, A T, Techner, F, and Thomas, K., *Proc Soc Exp Biol and Med*, 1932, 20, 609

<sup>2</sup> Freiberg, I K., and West, E S, *J Biol Chem*, 1933, 101, 449

† These cases were used by Freiberg and West in a study of glycine synthesis under benzoate stimulation. The writers are indebted to Dr C H. Crego, Jr, and his staff of the Shriners' Hospital for Crippled Children in St Louis for making these cases available, and for excellent cooperation in the study.

<sup>3</sup> Gibson, R. B., and Martin, T F, *J Biol Chem*, 1921, 40, 319

are large amounts of pentosan in bran. The purpose of this work was to compare the laxative properties of these 2 nondigestible carbohydrates, and also to determine whether the laxative effect was due entirely to the physical property of increasing the amount of unabsorbable matter and water content of the stool, or whether the metabolic products formed by the action of intestinal bacteria on them might be stimulating to the intestine. The volatile fatty acids are one of the principal split products of the action of bacteria on both digestible and nondigestible carbohydrates. We have already shown them to be greatly increased when assimilable carbohydrate predominates in the diet.<sup>1</sup>

The plan of the work was to use 2 human subjects whose colons appeared normal by X-ray studies. Four feeding periods each of 7 days. The first period consisted of feeding a nonresidue basal diet, in the second period the basal diet was supplemented with 35 gm daily of a crude preparation of pentosan from bran which contained 40% pentosan by weight, in the third period the basal diet was supplemented with 35 gm daily of a fraction of bran containing 50% crude fibre, and in the fourth period apples, apricots, and prunes were added to the basal diet.

The stools were weighed immediately and steam sterilized and sealed in fruit jars. They were analysed for pentose by determining the nonfermentable sugar after acid hydrolysis and precipitated by mercuric sulphate. The fibre was determined by the usual Weender method as outlined in official methods of Association of Agricultural Chemists. Volatile acids by our own method.<sup>2</sup>

Of the pentosan fed, only 17% in one subject and 6.5% in the other was recovered in the stools. In contrast to these results, 102% and 95% of the fibre fed was recovered. The marked destruction of pentosan did not increase either the volume of the stools or the output of volatile fatty acids. When, however, fibre was fed, the volume of the stools increased in one subject 100% and in the other 88%. The volatile fatty acids also increased 56% in one subject and 74% in the other. During the period when fruits were added to the basal diet there were definite increases in the stool volumes and amounts of volatile fatty acids.

*Conclusions* The pentosans of bran are not laxative while bran fibre is definitely so. The volatile fatty acids parallel the volume of the stool and are not increased by the breakdown of pentosan.

<sup>1</sup> Grove, E. W., Olmsted, W. H., Koenig, Karl, *J. Biol. Chem.*, 1929, **85**, 127.

<sup>2</sup> Olmsted, W. H., Duden, C. W., Whitaker, W. M., Parker, R. F., *J. Biol. Chem.*, 1929, **85**, 115.

observation showed little change with possibly some further degeneration. The creatine and creatinine excretions also indicate that the condition did not improve and probably became worse. Notwithstanding these facts, the general condition of the patients appeared some better during the gelatine feeding.

Fifteen gm of glycine were fed daily beginning 7-16-'33, and continuing for 3 months in the case of M S, 2 months in the case of D G and  $2\frac{1}{2}$  months in the case of C E. The characteristic increase in creatine excretion was observed, the increase being little greater than caused by 28 gm of gelatine (equivalent to about 7 gm of glycine). This suggests that constituents in gelatine other than glycine caused considerable of the increased creatine excretion. The glycine feeding had no apparent beneficial effect.

The peculiar muscular sensations noted by Milhorat, Techner and Thomas during glycine feeding were not observed at any time in our cases.

The administration of ephedrine sulfate (4 doses of  $\frac{3}{8}$  grain at 3 hour intervals) to the cases while on a meat-free diet did not significantly change the creatine or creatinine excretion. Reinhold and others<sup>5</sup> reported the creatine excretion of a dystrophy case (type of dystrophy uncertain) as doubled by ephedrine administration while receiving glycine.

D G excreted 72% of 10 gm of creatine when ingested after breakfast on a meat-free diet. C E and M S excreted 87 and 65% respectively.

*Conclusions* Cases of pseudohypertrophic progressive muscular dystrophy seemed to be somewhat improved, as judged by exercise tests, by prolonged gelatine feeding. The improvement, if any, was of a temporary nature and the progress of the condition was not arrested. The creatinine coefficients were lower at the end of the gelatine feeding than before and the creatine excretion showed little change. We believe that gelatine feeding affords as much stimulation as glycine feeding in this condition.

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<sup>5</sup> Reinhold, J G, Clark, J H, Kingsley, G R, Custer, R P, and McConnell, J W, *J A M A*, 1934, 102, 261

feeding The creatine excretion on a meat-free and gelatine-free diet after the period of gelatine feeding was essentially as in the control period before gelatine feeding The creatinine coefficients before and after feeding gelatine were for C E, 3.7 and 1.76†, for M S, 2.9 and 1.66, and for D G, 3.5 and 2.6 respectively, representing a definite decrease in each case Harris and Brand<sup>4</sup> have pointed out the correlation of a low creatinine coefficient and the severity of the disease We have observed the same thing in 3 other cases (boys in the same family), in which the creatinine coefficients were inversely proportional to the severity of the condition

TABLE I  
C E, male, age 11 years \* Could not walk or rise from sitting position Muscular weakness from infancy progressively becoming worse

Periods days	Diet	Creatinine Mg 24 hr Aver	Creatino Mg 24 hr Aver	Exercise‡ Slud Feet in min
24†	No meat, no gel	247	400	182
24	" " 18 gm gel	233	527	161
13	" " 28 " "	243	790	364
16	" " 42 " "	214	808	298
3	" " 28 " "	246	692	1456
7	" " no gel	247	641	1820
18	" " 28 gm gel	243	698	1456
14	Same	230	672	364
13	" "	237	713	1456
17	" "	191	660	546
17	No meat, no gel	157	467	1638
24	Same	172	401	1350
20	Hospital diet	214	508	1456
13	No meat, no gel	193	459	455

\*Weight increased progressively from 25 kilos on 7 12 32 to 36 kilos on 3 30 33  
Much of the increase was due to fat

†Began 7 13 32 Periods continuous until 2 10 32, with the exception of a day or two between some of the periods 20 days elapsed between periods 11 and 12, but the diet was unchanged 2½ months elapsed between periods 13 and 14.

‡Exercise tests were given at intervals of 3 to 4 weeks, and included the interval between 9 22 32 and 10 11 33

The last column of Table 1 gives the results of exercise tests on C E, throughout and following gelatine feeding Such tests have serious disadvantages, yet probably indicate something as to relative muscular ability As judged by this test all showed considerable improvement during the gelatine feeding and 2§ maintained it for sometime after gelatine was discontinued

Clinical examination of the muscles throughout the period of

† He had become quite fat during the experiment

4 Harris, M. M., and Brand, E, *J A M A*, 1933, 101, 1047

§ D G became unable to walk during April of 1933 Gelatine feeding was discontinued 1 24-33 in all cases

discussion on the relation of heredity to duration of life, Pearl<sup>6</sup> states, it "Indicates that from one-half to three-fourths of the death rate is selective in character, because that proportion is determined by hereditary factors Just in proportion as heredity determines the death rate so is the mortality selective" Obviously the known relation of heredity to longevity, whatever its magnitude may be, has a definite place in the problems of human constitution. But before this relation can be useful in these problems, the types of inherited characters must be shown to remain permanent, or relatively so, after type differentiation, and their possible age incidences must be investigated. *The only investigations of inherited characters in relation to age and longevity, recorded in the literature, are those on human scapulae*

A brief summary of the writer's studies based on human scapulae will indicate the possibilities of similar studies of other inherited characters and among these, blood groups Incident to family studies in 1906, he was led to classify the scapulae of man and some other mammals into *convex* and *scaphoid* (straight, concave and mixed) types Observing in 1907, that *convex* types predominate in the *old* and *scaphoid* types in the young, he was led to investigate the origin, permanence, distribution and age incidence of scapular types, as well as their possible relation to longevity<sup>7-20</sup> His investigations show that, in man, the range in variation of scapular types is from the extremely *convex* through the *straight* to the extremely *concave*, their origin is primal, in man, they are transmitted with unusual constancy, regardless of sex, the *scaphoid* (straight, concave and mixed) types as *dominants*, they are differentiated in man in pre-natal life, they remain *permanent* in type in man throughout the life span, regardless of ageing processes, nutrition, health, disease, oc-

<sup>6</sup> Pearl, R., *The Biology of Death*, p 177, Philadelphia and London, 1922

<sup>7</sup> Graves, W W, *Med Record* (N Y), 1910, 78, 861

<sup>8</sup> Graves, W W, *Trans Nat Assn. for Study of Epilepsy and Care and Treatment of Epileptics*, 1911, 8, 56

<sup>9</sup> Graves, W W, *Contrib Med and Biol Research*, dedicated to Sir William Osler, 1919, 1, 525

<sup>10</sup> Graves, W W, *Am J Physiol Anthropol*, 1921, 4, 111

<sup>11</sup> Graves, W W, *Am J Phys Anthropol*, 1922, 5, 21

<sup>12</sup> Graves, W W, *Trans Am Assn. of Life Ins. Med Directors*, 1923 (Discussion Dublin, L L, Rogers, O H., Patton, J A, Hoffman, F L, and Graves, W W)

<sup>13</sup> Graves, W W, *Arch Int Med*, 1924, 34, 1

<sup>14</sup> Graves, W W, *Z f Konstitutionen*, 1925, 11, 717

<sup>15</sup> Graves, W W, *Arch Int Med*, 1925, 36, 51

<sup>16</sup> Graves, W W, *Glasgow Med J*, 1925, 315

## Possible Relation of Blood Groups to Age and Longevity

WILLIAM WASHINGTON GRAVES

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The writer's purpose is to point out hitherto unrecognized possibilities of blood-group investigations in relation to the problems of human constitution

Landsteiner found that blood-group formation is physiological, independent of pathological processes<sup>1</sup> This discovery and the findings of von Dungern and Hirszfeld<sup>2</sup> that groups A and B are transmitted as *dominants*, stimulated much additional research on the problems of serology, heredity, anthropology, paternity and constitution Hirszfeld<sup>3</sup> has stressed the importance of further research on the relation of blood groups to the problems of human constitution

The problems of constitution deal primarily with those innate characters which largely preserve individual identity, therefore only those characters which are classifiable into discernible types in the living, and which, after type differentiation, remain permanent, or relatively so, throughout the life span, may be useful in these problems The results of all investigations show that blood groups are innate and indicate that, after type differentiation early in life, the group inherited by the person remains permanent, regardless of disease and other environmental influences,<sup>4</sup> <sup>5</sup> hence further research on the relation of blood groups to problems of human constitution may be of great promise, although the recorded results, thus far, are very contradictory May not the reasons for such results be found in the lack of uniformity in technical details, race, stock or even community differences, inadequate numbers and controls and *possibly in the fact that blood groups have not yet been studied in relation to age and longevity?*

The relation of heredity to longevity has long been recognized Genealogical and other studies show that longevity is heritable not only in man, but in lower forms<sup>6</sup> In summing up his mathematical

<sup>1</sup> Landsteiner, K, *Wien klin Woch Bd*, 1901, 14, 1132

<sup>2</sup> v Dungern, E, und Hirszfeld, L, *Z f Immunitäts*, 1910, 6, 284

<sup>3</sup> Hirszfeld, L, *Ergeb Hyg Bakteriol*, 1926, 8, 366

<sup>4</sup> Lattes, L, *Die Individualität des Blutes*, Trans by Schiff, F, Berlin, 1925

<sup>5</sup> Pearl, R, *The Rate of Living*, pp 1 18, New York, 1928

of Medicine and Sister Mary Francis, Technician, University Hospital. The investigations of Sister Mary Francis deal with healthy and with hospitalized and out-patient material (white males and females) in successive age periods from birth onward. The results of her investigations will be published elsewhere. Those of Pulley and Mitchell are with white male students in the Medical School and ambulatory white male inmates of the St. Louis Infirmary (Alms House). The results of their investigations thus far available, arranged according to the international classification, are shown in Table I.

TABLE I.

Age yrs.	Total	O		A		B		AB	
		No	%	No	%	No	%	No	%
22-26	281	115	40.9	112	39.9	38	13.5	16	5.7
60+	500	232	46.4	203	40.6	48	9.6	17	3.4
		% increase 13.9		% increase 1.7		% decrease 28.8		% decrease 40.4	

As far as known, the first investigations on the possible age incidence of blood groups are those of Pulley and Mitchell and of Sister Mary Francis. Pulley's and Mitchell's results, thus far, are based on admittedly small numbers and while the number and percentage differences in relation to age are comparable, they are not conclusive, even for the age periods represented. However, the known age incidence of scapular types and its explanation suggest the possibilities of further blood-group investigations in relation to age. Such investigations by many workers with large numbers, representing healthy and sick groups of different races or stocks and different communities of the same race or stock, may disclose definite answers to the questions: Is there an age incidence of blood groups? If so, can it, like that of scapular types, be explained on the known relation of heredity to longevity? Affirmative answers to these questions will enlarge the usefulness of blood groups in relation to the problems of human constitution, as these are expressed in innate predisposition to health or disease, innate capacities for living and adaptability in general.

*Acknowledgements* The writer wishes to thank Mr. H. C. Pulley and Dr. J. B. Mitchell, Jr., for the use of their figures and to express his appreciation of the encouragement given him by Professors Moyer S. Fleisher and John Auer in the effort to stimulate further research on the relation of blood groups to the problems of human constitution.

cupation and other environmental influences, they are found in varying percentages in the remains of ancient and modern man and of some other mammals (gorilla, orang, chimpanzee, armadillo, bat *et al*), they are present in varying percentages in the excellently, well and poorly adaptable, regardless of age, race, stock and body build, in similar age periods, they may be present in varying percentages in different communities of the same race or stock, and in skeletal material and in healthy and sick groups, representing successive age periods from childhood to old age, the percentages of *convex* types increase, while those of *scaphoid* types decrease. His more recent figures on the age incidence of scapular types in white stocks are approximately as follows: 6 to 15 years *scaphoid* types 65%, *convex* types 35%, 60 years and over *scaphoid* types 35%, *convex* 65%.

Since adequate investigations, including follow-up, lead to the conclusion that scapular types remain *permanent* throughout the life span, the only tenable explanation for their age incidence is *better adaptability, less morbidity, greater longevity among the bearers of the convex than among the bearers of the scaphoid types*. This explanation is supported by the writer's figures and those of others<sup>17, 19, 20</sup> showing that in the healthy and sick groups studied in similar age periods there are approximately from  $1\frac{1}{4}$  to  $2\frac{1}{4}$  times as many *convex* types in the healthy as in the sick groups. Whether the types of other inherited characters will show similar age incidences in relation to the problems of constitution (adaptability, morbidity and longevity) cannot be known until such types have been similarly investigated.

Since blood groups had not been investigated in relation to age and longevity, and since it seemed that investigations of their possible age incidence might reveal the presence or absence of such relation, investigations were begun in 1933 by Mr H C Pulley, Assistant, Department of Bacteriology and Dr J B Mitchell, Jr, Instructor, Department of Pharmacology, St Louis University School

<sup>17</sup> Graves, W W, The Relations of Shoulder Blade Types to Problems of Mental and Physical Adaptability, The Henderson Trust Lecture, No IV, Edinburgh, 1925

<sup>18</sup> Graves, W W, *Eugenics Rev*, 1931, 23, 215

<sup>19</sup> Graves, W W, A Note on Inherited Variations and Fitness Problems I. The Types of Scapulae. Trans Third Internat'l Congress of Eugenics, Baltimore, 1934.

<sup>20</sup> Graves, W W, The Relation of Inherited Variations of Structure and Function to Problems of Health, Disease, Education, Duration of Life, and Adaptability in General. I. The Types of Scapulae. (In publication.)



daily for 2 weeks previous to and for 6 days following pancreatectomy. There was no appreciable glycosuria ( 3 to 5 gm ) during the period following operation. However, when oestrin injections were suspended the urine sugar increased to 11 gm. When oestrin injections were resumed the urine sugar gradually decreased. In one animal the administration of 200 R. U. daily resulted in the total disappearance of sugar. Two animals received no oestrin prior to operation and were allowed to develop a glycosuria before injections were initiated. The injection of 100 R. U. daily resulted in the complete disappearance of sugar. Alternate periods of injections and withdrawal of injections resulted in corresponding fluctuations in the urine sugar. During one of the periods of treatment the animals also received injections of the pituitary extract. As a result sugar reappeared in the urine and persisted for several days. At the present time, over 5 months after operation, 3 of the 4 operated animals are alive and in good condition. The weight lost during periods when no oestrin was given has been more than compensated for under oestrin treatment. One monkey died 72 days after pancreatectomy. Her diabetes had been well-controlled according to blood and urine sugar findings. Autopsy showed the probable cause of death to be numerous metastatic abscesses in the liver and lungs from a pyogenic blood stream infection originating in an abdominal abscess present prior to operation. This abscess failed to respond to treatment and probably was chiefly responsible for the animal's death.

Blood sugar tests on normal fasting monkeys have shown a considerable range ( 55 to 125 mg per 100 cc ). Following pancreatectomy it has risen as high as 550 mg per 100 cc and has been regularly decreased by the administration of oestrin. However, in the 2 totally pancreatectomized animals we have been unable to lower the blood sugar level to the normal level. For example monkey 17 had a normal level of 125 mg, a diabetic level of 550 mg, and a level after oestrin treatment of 185 mg.

It is believed that the evidence presented here may be interpreted as evidence that oestrin has suppressed the diabetogenic activity of the anterior lobe. These studies are being continued and extended.

# Effect of Oestrin Injections upon Experimental Pancreatic Diabetes in the Monkey \*

WARREN O NELSON AND MILTON D OVERHOLSER (Introduced by A J Goldforb )

*From the Department of Anatomy, University of Missouri.*

The anterior hypophysis has been shown to be related to carbohydrate metabolism by the studies of Houssay and Biasotti<sup>1</sup> in which the acute diabetes induced by pancreatectomy, in the dog, was relieved by removal of the anterior hypophysis. In a study based on the recognized action of oestrin in suppressing the anterior lobe it was shown by one of us<sup>2</sup> that administration of oestrin in depancreatized dogs had much the same effect as removal of the hypophysis.

Repetition and extension of this work on a form more closely related to man was believed to be of some importance. Accordingly we have employed the rhesus monkey in a series of experiments designed not only to repeat the studies on the dog, but also to gather data concerning the physiological mechanisms involved, and to determine the effect of continued oestrin administration, in depancreatized animals, upon certain other organs. At this time we are reporting the results of experiments conducted on 6 immature female monkeys (3 to 3.5 kg. in weight).

In 2 animals the injection of an acid extract of beef hypophyses<sup>3</sup> has induced hyperglycemia and glycosuria. We have used, uniformly, the Shaffer-Somogyi<sup>3</sup> method for sugar determination and have determined the true sugar as well as total reducing substances in both blood and urine. Twenty-four hour collections of urine were made regularly.

Four animals have been depancreatized. In 2 cases a small fragment of pancreas was not removed. In the remaining 2 animals pancreatectomy is believed to have been complete.

Two animals were treated with 100 R. U. oestrin (Theelin†)

\* This investigation was aided by a grant from the Committee on Scientific Research of the American Medical Association to Dr. M. D. Overholser.

<sup>1</sup> Houssay, B. A., and Biasotti, A., *Endocrinology*, 1931, 15, 511.

<sup>2</sup> Barnes, B. O., Reagan, J. F., and Nelson, W. O., *J. Am. Med. Assn.*, 1933, 101, 926.

<sup>3</sup> Shaffer, P. A., and Somogyi, M., *J. Biol. Chem.*, 1933, 100, 895.

† The pituitary extract was supplied through the kindness of Dr. Oliver Kamm and Dr. D. A. McGinty of Parke, Davis and Company. The Theelin used in this experiment was an oil solution, also supplied by Dr. Kamm.

of epinephrine, 0.2 cc to 1 cc, hypodermically administered produced violent attacks with cyanosis, blanching, and pain. Attacks were relieved in 5 minutes by intravenous glucose. Fifteen units of insulin produced a severe attack in 50 minutes. The blood sugar dropped from 70 to 33 mg %. Intravenous glucose quickly relieved attack. No effect occurred in vessels of feet or of other parts not obviously abnormal during either of the tests.

Case II Male with well developed Raynaud's disease of upper extremities, gangrene of tips of 3 fingers on each hand. Response to epinephrine and insulin similar to that in Case I. Cyanosis after insulin extended almost to shoulders. The blood sugar dropped from 54 mg % to 27 mg %.

Case III Male with definitely established attacks of hypoglycemia in which blood sugar dropped to 14 mg %. During each attack patient's fingers blanched and caused him to experience burning pain. Removal of  $\frac{3}{4}$  of the pancreas with relief from attacks of hypoglycemia also resulted in relief from attacks of white fingers.

These 3 cases demonstrated a local hypersensitiveness of the blood vessels to circulating epinephrine. There was normal or increased sweating and no loss of pilomotor function in the diseased extremities. No operation on sympathetic nerves had been carried out in any of these patients. Previously such hypersensitiveness to epinephrine on the part of smooth musculature has been noted following denervation. Investigation as to the nature of changes in tissues responding paradoxically to epinephrine is, therefore, being continued, particularly from the point of view of nervous function.

That the nerve supply to blood vessels was functional in these cases was demonstrated as follows. Anesthesia of the nerves of the arm in Case I resulted in definite dilatation of the vessels and cooling curves taken in a water-jacketed plethysmograph indicated greater constriction (faster cooling) in the hand normally than after anesthetization. Case II developed pain in all the fingers of one hand in a plethysmograph when the air was 21°C, although a thermometer between 2 fingers gangrenous at their tips read 34°C. This occurred within 5 minutes after cooling started, the air in the room being 32°C, and the initial temperature between the fingers 33.5°C. Reflex constrictions resulting in pain, therefore, occurred before significant cooling. Dipping the elbow of either arm in water at 6°C resulted in pain in the fingers within 2 minutes homolaterally, but not contralaterally (local reflexes) with no measurable change in temperature of the palm or fingers. This pain persisted for 10 minutes with the arm in the air at 32°C, with cyanosis. The

## On the Mechanism of Spastic Vascular Disease \*

PETER HEINBECKER AND G H BISHOP

*From the Department of Surgery and the Laboratory of Neurophysiology, Oscar Johnson Institute, Washington University School of Medicine, St Louis*

Two opposing views exist as to the mechanism of spastic disease of blood vessels. Some regard it as an expression of dysfunction of the vaso-motor nerve supply to the vessels. Chief support for this conception is found in the symmetrical nature of the lesion and the paroxysms which characterize it. Others, especially Lewis and his coworkers, regard it as a local fault not primarily associated with abnormal innervation. This local defect as studied in Raynaud's disease of extremities expresses itself in an abnormal response to cold, in the spatial order of development and disappearance of the vascular constriction, and in the failure of local anesthetization of the nerve supply to prevent or release completely an attack. Our own observations on these aspects of the disease lead us to support the contention of Lewis and others.

Evidence is herein presented that the fault is a local one, and represents not a hyperfunction of a sympathetic innervation, but a change in the blood vessels, namely that they respond to epinephrine in a manner similar to tissues deprived of their sympathetic nerve supply (paradoxical response) while the nerve supply can be demonstrated to be functional.

The evidence is derived from the study of 3 patients with vascular abnormalities of the upper extremities, 2 cases of Raynaud's disease and one case of acro-asphyxia. The first 2 were subjected to the following tests: (1) 1 cc. or less (graded doses) of epinephrine hydrochloride (1-1000) was given hypodermically and the effect on the diseased and control extremities noted. An attack was invariably induced in the diseased extremities. Then the effect of intravenous glucose or of a meal rich in carbohydrate was noted. (2) Ten to 15 units of insulin were administered to produce a physiological secretion of epinephrine. The effect on the diseased and control extremities was noted and again the effect of carbohydrate on the attack determined.

Case I. Male, age 37 with well advanced Raynaud's disease of fingers of both hands, the tip of one finger was gangrenous. Doses

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\* Assisted by a grant in Aid of Research in Neurophysiology from the Rockefeller Foundation.

## Ovarian Irradiation and Sexual Precocity in the Rat

JAMES MANDEL AND E N GRISEWOOD (Introduced by H O Haterius)

*From the Departments of Biology and Physics, New York University*

Incidental to some work with X-rays the writers have had occasion to carry out a series of experiments to determine the effects of X-ray treatment upon the time of sexual maturity in the rat as evidenced by the criteria of vaginal canalization and appearance of an oestrous smear

Immature females, 7 to 10 days of age, were subjected to varying dosages of X-rays. One week later the same dosage in each case was repeated. The treatments were given under the following conditions: 200 k v p (meter reading), 3 milliamps, 25 cm target-to-specimen distance, 1 mm Cu filter. This resulted in a dosage of 41.4 roentgen units per minute as determined by a standard ionization chamber. The effective wave length, as found by the method of relative penetration of Al and Cu, was 13 Å U. Dosages ranged from 540 to 1,240 'r' units, amounts above the latter figure proved fatal, death occurring within from a few days to as long as several weeks after the first irradiation.

Striking results were obtained in all the irradiated animals in that, regardless of dosage within the limits indicated, the vaginae opened at approximately 14 days after the first irradiation, at an average litter age of 22 days. Vaginal smears in each instance revealed a characteristic oestrous picture a day or two later, and the uterus, in each instance in which examination was made, appeared markedly distended. Twenty-three animals, representing 11 litters, showed this response. Controls, at least 1 litter mate for each experimental animal, in each case displayed vaginal opening followed by oestrus at 43-60 days—at least 3 weeks later than the irradiated animals.

The X-rayed ovaries of animals sacrificed at time of vaginal opening, 22 days of age approximately, revealed the presence of huge follicles, with enormous antra. Few follicles of small size were to be found. The ovaries of litter mate controls presented a marked contrast in appearance in that only small and medium-sized follicles were in evidence. It appears that the X-ray treatment had stimulated the growth of follicles tremendously, and that these follicles have been functionally hyperactive as well as evidenced by the fact that vaginal canalization was markedly precocious.

patient was put in a cold room at  $6^{\circ}\text{C}$  with both arms bundled to the shoulder in 3 inches of cotton batting. Pains occurred while the temperature between the fingers was still rising (initial  $T$   $33.5^{\circ}\text{C}$ , pain at  $34^{\circ}\text{C}$ , intense pain at  $33.8^{\circ}\text{C}$ ) and on coming out of the cold room the pain stopped in 4 minutes with no detectable change of temperature in the fingers of the wrapped hand ( $33.8^{\circ}\text{C}$ ). The pain was obviously due to reflex spastic constrictions or circulating epinephrine and not to changes in temperature of the fingers. The alternative to the defect being a local one in the arteries would seem to be to suppose a hyperfunction of the nervous supply to these parts. This would not explain a paradoxical reaction to epinephrine.

These conditions could be explained by inferring a local (paradoxical) reaction to epinephrine or similarly acting body substances, but without the loss of nervous function which is the one known cause of such a condition. But the local reaction (spasm, pain) is not due only to circulating epinephrine, it can be called forth reflexly and also by local cooling of the anesthetized parts. That is, the affected parts go into spasm due to all the stimuli that would normally cause mild constriction, and pain and gangrene result secondarily from arterial spasm rather than from cold.

These findings may be applied to a better understanding of spastic vascular disease, such as Raynaud's disease of the extremities or certain cases of angina pectoris, etc., where external and internal alterations in environment are known to result in an increase in circulating constricting agents and also to produce 'attacks'. They cast doubt on the soundness of conception of surgical procedures in which the sympathetic nerve supply to blood vessels is interrupted. Procedures involving the removal of post-ganglionic neurones would tend especially to exaggerate to maximum degree an already existing fault. Procedures resulting in the interruption of pre-ganglionic neurones would exaggerate the fault to a lesser degree because the fully developed paradoxical response to epinephrine occurs only after removal of post-ganglionic neurones. Such procedures, while resulting in hyperaemia and in the relief of pain, may do so only temporarily until the aggravation of the condition due to denervation leaves the remedy more embarrassing than the original disease.

Uncomplicated arteriosclerosis to the extent of producing gangrene of the extremities does not involve anything resembling the paradoxical epinephrine reaction.

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# Studies on the Neutral Sulfur of Urine Criticism of the Iodimetric Titration of Diethylsulfide \*

GRACE MEDES, KIVELY EVANGELIDES AND KAMENOSUKE SHINOHARA †

Abel<sup>1</sup> found diethylsulfide in dogs' urine which had been treated with calcium hydroxide Christomanos<sup>2</sup> determined the amount of the sulfide in urine by iodimetry, basing his method upon the formation of the addition product of diethylsulfide and iodine,  $(C_2H_5)_2SI_2$ , previously reported by Cahours<sup>3</sup>

TABLE I.  
Iodine taken up by  $Et_2S$  from  $I_2$  solutions, with variation in the different components of the system.

Conc KI mols per l $\times 10^2$	Conc $I_2$ mols per l $\times 10^2$	Conc HI mols per l $\times 10^2$	Conc H ions* mols per l $\times 10^2$	$Et_2S$ Mols $\times 10^3$ added to 1 l sol	$I_2$ Mols $\times 10^3$ taken up	Ratio $\frac{I_2}{Et_2S}$
2.4	1.0			1.46	3.09	2.16
6.0	1.0			0.96	1.22	1.27
18.0	1.0			1.34	0.25	0.86
24.0	1.0			1.27	0.21	0.17
6.0	0.265			1.32	0.15	0.11
6.0	0.505			1.00	0.35	0.35
6.0	0.790			0.92	0.72	0.70
6.0	1.00			0.96	1.32	1.27
6.0	2.61			1.23	3.13	2.56
6.0	2.0			6.66	10.55	1.59
6.0	2.0			16.48	17.48	1.06
6.0	2.0			20.52	18.28	0.89
6.0	2.0			24.22	18.89	0.78
6.0	2.0			35.58	19.45	0.55
	2.0	8.0		1.83	2.80	1.53
	2.0	8.0		6.55	9.47	1.46
	2.0	8.0		17.95	16.77	0.93
	2.0	8.0		24.30	18.42	0.76
	2.0	8.0		34.61	19.00	0.55
	2.0		8.0	1.85	2.80	1.53
	2.0		6.0	1.81	2.76	1.52
	2.0		5.0	2.03	2.95	1.45
	2.0		3.0	2.27	3.22	1.42
	2.0		1.0	2.00	2.75	1.38
	2.0		0.0	1.80	2.29	1.27

\*Hydrogen ion concentration was calculated by assuming the hydrogen iodide to be totally ionized 0.04N iodine solutions in 0.08M KI and 0.08M HI were combined in various ratios so that the total concentration of electrolyte might remain constant.

\*Aided by a grant from the Leffman Fund of the Wagner Free Institute of Science, Philadelphia

† Robert McNeill Fellow of the McNeill Laboratories

1 Abel, J. J., *Z. physiol. Chem.*, 1896, **20**, 253

2 Christomanos, A. A., *Z. physiol. Chem.*, 1933, **217**, 177

3 Cahours, *Ann. d. chim. et d. phys.* 1865, **135**, 355



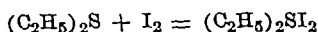
The authors have been unable to confirm this stoichiometric relationship under the conditions described by Christomanos and investigated the factors influencing the distribution of iodine in the system, diethylsulfide—aqueous solutions of potassium iodide

The experiments were carried out as follows. The diethylsulfide was weighed by difference from a weighing burette into 500 cc volumetric flasks embedded in an ice-water mixture ( $t = 2^{\circ}$  to  $5^{\circ}$ ) and containing iodine, potassium iodide and hydrogen iodide. The mixtures were shaken until equilibrium was reached with respect to the iodine distribution. A portion of the mixture was centrifuged at  $2^{\circ}$  to  $5^{\circ}$ , and 25 cc of the clear aqueous solution were subjected to iodimetric titration. The results are shown in Table I.

*Results* The amount of iodine taken up by diethylsulfide varies with the amount of the sulfide and also with the concentration of iodine, iodide ion and hydrogen ion, the effect of the last factor being the slightest. This suggests that the removal of iodine from its solution by diethylsulfide is due to the solubility of iodine in the sulfide. In fact, iodine was found to be miscible with diethylsulfide roughly in all proportions.

In the light of the present experimental results the method for the determination of diethylsulfide in biological solutions should, therefore, be based upon other principles.

*Summary* The amount of iodine taken up at a constant temperature by diethylsulfide is the function of the concentrations of iodine, iodide ion and hydrogen ion in addition to the amount of diethylsulfide added, and there is no such chemical relation under the experimental conditions as expressed by the equation



The analytical method based upon this erroneous principle is unreliable.

7597 C

### Effects of Avian Pituitary Glands in Salamanders

KATHRYN F. STEIN (Introduced by A. E. Adams)

*From the Zoology Department, Mount Holyoke College*

Induction of ovulation has been secured in various forms by administration of implants or extracts of the pituitary glands of

species and of classes other than that of the host animal<sup>1-7</sup> Response of the host was negative, however, in the following cases pigeon implants in mice,<sup>1</sup> implants of hypophyses of cow, dog, guinea-pig, rat, frog, fish, chicken, and snake under the skin of toads,<sup>3</sup> frog implants and mammalian implants and extracts in toads,<sup>7</sup> frog hypophyses in mice,<sup>8</sup> and in toads,<sup>9</sup> rat implants in toads<sup>10</sup> Further experiments indicated that the probable explanation of these negative results, at least in some cases, was failure to administer the glands in adequate dosages Thus by increasing the daily dosage Lipschutz, Kallas and Wilckens<sup>11</sup> were able to induce ovulation in mice with pigeon pituitaries, and Wills, Riley and Stubbs<sup>10 12</sup> secured positive results in toads with frog and fish glands The latter authors therefore concluded that, contrary to the conception of Houssay *et al*,<sup>3</sup> there was no specificity of the maturity hormone of the anterior lobe of the pituitary among anurans Their subsequent failure to obtain ovulation in toads by rat implants caused them to admit a possible specificity between these classes, with the alternative that the hormone might be destroyed due to the incompatibility of the tissues and subsequent reactions<sup>10</sup> The following experiments, in which avian anterior lobes were administered to urodele hosts, do not support the idea of specificity of hormones and are reported as an additional case of induction of ovulation by heteroplastic implants and injections of pituitary They are also concerned with the difference in potency between glands of young and adult donors, and with the effect of implants and injections on the thyroid of the host animal

*Implants of Adult Fowl Pituitaries* Glands were obtained from fowl (chiefly Rhode Island Red roosters) killed for market The

1 Smith, P E, and Engle, E T, *Am J Anat*, 1927, 40, 159

2 Zondek, B, und Aschheim, S, *Arch f Gynakol*, 1927, 130, 1

3 Houssay, B A, Giusti, L, et Lascano-Gonzalez, J M, *Compt Rend Soc Biol*, 1929, 102, 864

4 Adams, A E, *Proc Soc Int Cong for Sex Res*, 1930, 190

5 Kehl, R, *Compt Rend Soc Biol*, 1930, 103, 744

6 Adams, A E, *Anat Rec*, 1931, 49, 37

7 Adams, A E, *Proc Soc Exp Biol and Med*, 1931, 28, 877

8 Lipschutz, A, and Pacz, R, *Compt Rend Soc Biol*, 1928, 99, 693

9 Bardeen, H W, *Proc Soc Exp Biol and Med*, 1932, 29, 846

10 Wills, I A, Riley, G M, and Stubbs, E M, *Proc Soc Exp Biol and Med*, 1933, 30, 784

11 Lipschutz, A, Kallas, H, and Wilckens, E, *Comp Rend Soc Biol*, 1929, 100, 28

12 Wills, I A, Riley, G M, and Stubbs, E M, *Proc Soc Exp Biol and Med*, 1933, 30, 411

heads were kept on ice until needed when the pituitaries were removed and implanted in the back muscle of *Triturus viridescens* females. Implants were made from the same series of heads over a period of one week. Three animals were used as hosts and each received a definite third of the anterior lobe of each pituitary (Table I). Since 2 implants were made twice a day, each host received the equivalent in amount of approximately  $1\frac{1}{3}$  glands daily.

TABLE I.  
Adult Fowl Anterior Lobes in *Triturus* Females.

Animal	Daily Dosage	Total Implants	Duration	No of Eggs
CC1	2( $2 \times \frac{1}{3}$ ) glands	Ant. $\frac{1}{3}$ s $\approx$ $8\frac{2}{3}$ s glands	6½ days	None
CC2	"	Mid. $\frac{1}{3}$ s $\approx$ $6\frac{2}{3}$ s "	5 "	10 laid
CC3	"	Post. $\frac{1}{3}$ s $\approx$ $8\frac{2}{3}$ s "	6½ "	7 in oviducts

No eggs were ovulated by control animals kept under the same conditions while one of the 3 experimental animals laid eggs and a second had eggs in the oviduct when killed on the eighth day. The failure of CC1 to ovulate is not necessarily due to the region of the gland implanted but is more likely an individual difference in sensitivity to the hormone.

*Injections of Powdered Fowl Pituitaries* The anterior lobes of 35 pituitary glands from heads of Rhode Island Red roosters were treated with several changes of acetone, ground in a mortar, and the dry powder taken up in 0.6% salt solution. Injections of  $\frac{1}{2}$  to 1 cc were made daily into each of 2 female *Triturus* from October 18th to 23rd inclusive. One of the host animals laid eggs after 2, the other after 5 injections. No estimate can be made of the glandular equivalent of the injected material as the larger particles would not pass through the injecting needle and were allowed to settle out. At least 31 eggs were ovulated by one female and at least 32 by the other before the termination of the experiment on the sixth day. As ovulation did not occur in control animals during this period, it may be concluded that the anterior lobe of the fowl pituitary possesses gonad-stimulating hormone, the potency of which is not destroyed by acetone.

An attempt was made to extract fowl anterior lobes in pyridine following drying in acetone, but the potency of the material was apparently destroyed at some point in the procedure as no eggs were ovulated by either of 2 females given 11 injections from October 25th to November 5th inclusive.

*Implants of Chick Pituitaries* Rhode Island Red chicks, varying in age from one to 10 days, were decapitated and the anterior lobes of the pituitaries removed and implanted into the back muscle of 11

species and of classes other than that of the host animal<sup>1-7</sup> Response of the host was negative, however, in the following cases pigeon implants in mice,<sup>1</sup> implants of hypophyses of cow, dog, guinea-pig, rat, frog, fish, chicken, and snake under the skin of toads,<sup>3</sup> frog implants and mammalian implants and extracts in toads,<sup>7</sup> frog hypophyses in mice,<sup>8</sup> and in toads,<sup>9</sup> rat implants in toads<sup>10</sup> Further experiments indicated that the probable explanation of these negative results, at least in some cases, was failure to administer the glands in adequate dosages Thus by increasing the daily dosage Lipschutz, Kallas and Wilckens<sup>11</sup> were able to induce ovulation in mice with pigeon pituitaries, and Wills, Riley and Stubbs<sup>10, 12</sup> secured positive results in toads with frog and fish glands The latter authors therefore concluded that, contrary to the conception of Houssay *et al*,<sup>3</sup> there was no specificity of the maturity hormone of the anterior lobe of the pituitary among anurans Their subsequent failure to obtain ovulation in toads by rat implants caused them to admit a possible specificity between these classes, with the alternative that the hormone might be destroyed due to the incompatibility of the tissues and subsequent reactions<sup>10</sup> The following experiments, in which avian anterior lobes were administered to urodele hosts, do not support the idea of specificity of hormones and are reported as an additional case of induction of ovulation by heteroplastic implants and injections of pituitary They are also concerned with the difference in potency between glands of young and adult donors, and with the effect of implants and injections on the thyroid of the host animal

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<sup>4</sup> Adams, A E, *Proc Sec Int Cong for Sex Res*, 1930, 190

<sup>5</sup> Kehl, R, *Compt Rend Soc Biol*, 1930, 103, 744

<sup>6</sup> Adams, A E, *Anat Rec*, 1931, 49, 37

<sup>7</sup> Adams, A E, *Proc Soc Exp Biol and Med* 1931, 28, 677

<sup>8</sup> Lipschutz, A., and Paëz, R., *Compt Rend Soc Biol*, 1928, 90, 693

<sup>9</sup> Bardeen, H W, *Proc Soc Exp Biol and Med*, 1932, 29, 846

<sup>10</sup> Wills, I A., Riley, G M., and Stubbs, E M., *Proc Soc Exp Biol and Med*, 1933, 30, 784.

<sup>11</sup> Lipschutz, A., Kallas, H., and Wilckens, E., *Comp Rend Soc Biol*, 1929, 100, 28

<sup>12</sup> Wills, I A., Riley, G M., and Stubbs, E M., *Proc Soc Exp Biol and Med*, 1933, 30, 411

and stained in iron hematoxylin and Mallory B, and compared as to degree of stimulation. The thyroids of animals with *Triturus* and with adult fowl implants presented a picture similar to that obtained in *Triturus* by Adams<sup>13</sup> following injection of phyone and hebin for short periods. The height of the epithelium was increased over that found in normal glands, there were chromophobe vacuoles bordering the colloid indicative, according to Severinghaus,<sup>14</sup> of colloid absorption, and droplets of colloid within the cells. Implants of chick pituitaries likewise caused stimulation of the thyroids, slightly greater after 7½ glands than that found in normal or muscle-injected controls, markedly greater after 118 glands and after injection of powdered fowl glands in 0.6% salt solution. After both of the latter types of treatment, the thyroid picture resembled that obtained by Adams<sup>13</sup> after long-continued injection of phyone and hebin with hypertrophy and hyperplasia of the gland, high follicular epithelium, scanty colloid in the follicles, and increased vascularity. Practically all the glands possessed the type of active follicle described by Severinghaus<sup>14</sup> in the duck and characterized by high active epithelium on one side, and lower, inactive appearing cells on the other.

The fact that an hypophysectomized animal injected with chick anterior lobe molted after having received 14 glands also suggested that its thyroid was affected, since Adams *et al.*<sup>15</sup> have demonstrated that thyroidectomized animals will molt after thyroid but not after pituitary implants. Histological examination of the thyroid of this animal gave evidence of stimulation in that some of the epithelial cells were cuboidal rather than flat and apical vacuoles were present in the cells of some follicles, indicating colloid absorption. Such evidence was lacking in the thyroids of hypophysectomized controls.

**Summary** Anterior lobes of pituitaries of adult fowl (as fresh implants or powdered and injected in salt solution), or of young chick pituitaries (if a sufficient number of glands is implanted daily), will cause ovulation out of season in *Triturus viridescens* females. The thyroids of these females are stimulated beyond the normal condition. These results indicate that no specificity of hormone or hormones in ovulation-inducing and thyroid-stimulating capacities exists between birds and amphibians.

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<sup>13</sup> Adams, A. E., *Anat. Rec.*, 1934, **59**, 349.

<sup>14</sup> Severinghaus, A. E., *Z. f. Zell u. mik. Anat.*, 1933, **19**, 653.

<sup>15</sup> Adams, A. E., Kuder, A., and Richards, L., *J. Exp. Zool.*, 1932, **63**, 1.

TABLE II  
 Chick Anterior Lobes in Triturus Females

Animal	Daily Dosage	Total Implants	Duration	No of eggs
CH1	$\frac{1}{2}$ gland	$7\frac{2}{3}$ glands	22 days	None
CH2	"	$7\frac{1}{2}$ "	22 "	"
CH3	"	$12\frac{1}{2}$ "	32 "	2 in ovid
CH4	"	$11\frac{1}{2}$ "	32 "	None
CH5	"	11 "	32 "	"
CH6	"	$11\frac{1}{2}$ "	32 "	"
CH7	$2 \times \frac{1}{2}$ glands	44 "	23 "	"
CH8	"	44 "	23 "	"
CH9	$2 \times \frac{1}{2}$ (22 days)	50 "	53 "	6 laid
	$2 \times 1$ (3d), $2$ (3d)			7 in ovid.
	$2 \times 2$ (4d)			
CH10	$2 \times \frac{1}{2}$ (22d)	118 "	70 "	1 laid
	$2 \times 1$ (3d), $2$ (5d)			3 in ovid
	$2 \times 2$ (8d)			
	$2 \times 3$ (8d)			
Hypo'sec tonized	$2 \times \frac{1}{2}$ (2d)	14 "	14 "	14 in ovid
	$2 \times 1$ (4d), $2$ (2d)			

*Triturus viridescens* females (Table II) In 4 cases ovulation occurred, while none of a control series ovulated. The fact that 3 of the 4 pituitary-treated newts that ovulated received at least 2 glands per day at some time during the experiment, while only one (CH3) out of 8 receiving daily from one-half to one gland ovulated, seems to indicate that the latter may have been too small a daily dosage to cause ovulation except in very sensitive host animals. This is in line with the results, mentioned earlier in this paper, obtained after implants of pigeon pituitaries in mice and of frog pituitaries in toads, where a relatively small daily dosage gave negative, a larger, positive results. The need for the larger amount of chick pituitary might result from destruction of hormone by the host, failure of the tissue to release the hormone, or, and this seems more likely in the light of the results from adult glands, to the slight gonad-stimulating potency in the glands of young donors.

As a check, a series of 11 hosts were given implants of *Triturus* pituitaries and in every case ovulation occurred after 3 to 15 implants (average 7.5). Thus both the *total amount* and the *amount per dose* of *Triturus* gland tissue required for ovulation in *Triturus* were less than the *amounts* of fresh chick or fresh fowl tissue required. The average of the *total number* of glands of *Triturus* and of adult fowl was almost exactly the same, (7.5 *Triturus*, 7.6 fowl), but this may be merely a coincidence. Certainly the number of animals concerned is too small to draw any conclusion on this point.

*Effect of Avian Pituitaries on Thyroid of Triturus* The thyroids of control animals and of hosts receiving *Triturus*, chick, powdered fowl, and fresh fowl anterior lobes were fixed in picro-aceto-formol

finding renders it difficult at present to accept the opinion of some writers<sup>3</sup> that the *in vitro* lytic element observed by Tillett and Garner is an essential factor in accounting for the delayed fixation of a dye demonstrated by the author in the streptococcal type of inflammation in rabbits. It is possible that the fibrinolytic effect of hemolytic streptococci on the human plasma clot plays a definite rôle in human infections, but this is rather in the nature of a reinforcing or accessory factor added presumably to a more fundamental property of the hemolytic streptococcus which holds true for both the human and rodent types of infection.

In an attempt to analyze the problem further, experiments were performed with Berkefeld filtrates of the various types of pyogenic organisms previously studied. The details of this investigation will form the subject of a future communication, but the essential findings are summarized as follows:

- 1 The sterile filtrate of a several day old culture of *Staphylococcus aureus* induces an intense inflammatory reaction in the dermis of rabbits indistinguishable from the reaction obtained when the viable organisms are inoculated. Trypan blue is fixed as early as one hour after injection of the filtrate. Microscopic sections through the inflamed areas reveal many lymphatics occluded by a fibrinous reticulum. The tissue spaces are in many regions distended by coagulated plasma.

- 2 The staphylococcal filtrate is inactivated when heated for about one hour and a half at 58°C, for its cutaneous injection causes no fixation of the dye. Histologically, the lymphatic vessels are found to be entirely patent.

- 3 *Staphylococcus aureus* filtrate in contact with leucocytes obtained from an exudate causes these cells to become swollen, vacuolated, and, in many instances, degenerated. There is also some evidence that the total leucocyte count is lowered when this bacterial filtrate is maintained for some time in contact with an exudate. No such effect on leucocytes is produced with the heated and therefore inactivated staphylococcal filtrate. These observations strongly suggest that the active principle in the filtrate of *Staphylococcus aureus* which causes early blockage is somewhat similar, if perhaps not identical, to leucocidin.

- 4 On the other hand, the filtrates of both *Streptococcus hemolyticus* and Type I *pneumococcus* fail to induce retention of trypan blue at the site of cutaneous inoculation. There is no evidence of fixation even as late as 50 hours after skin injection of the streptococcal filtrate.

- 5 The strain of *Streptococcus hemolyticus* (S-23) used in all

## Further Studies on Mechanism of Invasiveness by Pyogenic Bacteria

VALY MENKIN

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The writer has recently demonstrated that the dissemination of a foreign substance from its site of inoculation is at least in part a function of its irritating capacity<sup>1 2</sup> *Staphylococcus aureus* induces a lesion sufficiently intense to occlude draining lymphatics and to cause the formation of a fibrinous network as early as one hour after cutaneous inoculation of the organism. The degree of "walling-off" was determined by studying the dissemination of trypan blue from the site of injury to the regional lymphatics. In the case of Type I *pneumococcus* the area is circumscribed at a somewhat later stage (about 6 hours subsequent to the inoculation of the organism). When *Streptococcus hemolyticus* is inoculated into the skin the lymphatics maintain their functional patency for about 2 days, and throughout that time these vessels are virtually unoccluded by thrombi. This histological observation adequately accounts for the delayed fixation and consequently the relative ease with which the dye penetrates to the regional lymphatics in a hemolytic streptococcal inflammation.

These results, while offering an explanation for the well-known localizing tendencies of the staphylococcus as against the disseminating properties of streptococcus present an interesting paradox. Staphylococci tend to remain localized and produce relatively slight systemic effects because of their pronounced local injurious action which serve to fix them *in situ*. Hemolytic streptococci, on the contrary, produce far greater systemic sequelae owing to the invasiveness resulting from their relatively mild local effects.

The experiments of the writer have recently been confirmed by Dennis and Berberian.<sup>3</sup> Subsequent to the writer's observations, Tillett and Garner<sup>4</sup> have demonstrated that broth cultures of hemolytic streptococci are capable of liquefying the normal human fibrin clot. In contrast to this they pointed out that the normal rabbit fibrin clot is totally resistant to dissolution by such means. This

<sup>1</sup> Menkin, V, *J Exp Med.*, 1933, **57**, 977

<sup>2</sup> Menkin, V, *Arch Path*, 1931, **12**, 802

<sup>3</sup> Dennis, E W, and Berberian, D A, *Proc Soc Exp Biol. and Med.*, 1934, **31**, 976

<sup>4</sup> Tillett, W S, and Garner, R. L., *J Exp Med*, 1933, **58**, 485



ting in 10 seconds Prothrombin must be the constituent that is removed or inactivated by aluminum hydroxide Significantly, this reagent does not remove or inactivate thrombin

Hirudin is a type of true antithrombin Heparin likewise appears to be an antithrombin as the following experiment demonstrates

Fibrinogen 0.5% solution (cc)	0.1	0.1	0.1	0.1	0.1
Fresh serum (cc.)	0.1	0.1	0.1	0.1	0.1
Heparin (mg)	0.0	0.003	0.006	0.009	0.012
Clotting time (sec)	15	60	300	No clot	

Heparin is not neutralized by thromboplastin Even when excess thromboplastin is present, the clotting time is prolonged as the concentration of heparin is increased Plasma containing 0.2 mg of heparin per cc will not clot, irrespective of the excess of thromboplastin

Other substances, notably certain dyes, are antithrombic. Calcomine Fast Pink 2 B L Unbl \* is a strong anticlotting agent

Fibrinogen 0.5% solution (cc)	0.1	0.1	0.1	0.1
Fresh serum (cc)	0.1	0.1	0.1	0.1
Calcomine fast pink (mg)	0.0	0.01	0.03	0.10
Clotting time (sec)	10	15	30	300

There is no evidence that the presence of thrombin in the blood stimulates the production of antithrombin. On injecting 70 cc of freshly defibrinated blood containing a high concentration of thrombin into dog (Body weight 13 kg) no intravascular clotting occurred, but free thrombin was still present in the blood 40 minutes after the injection On withdrawing 9 cc of blood and mixing it with 1 cc of M/10 sodium oxalate (an amount amply sufficient to prevent normal blood from clotting) a solid clot was formed in less than 24 hours by merely allowing the blood to stand This must be attributed to the free thrombin still present.

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\* The dye, Calcomine Fast Pink 2 B.L. Unbl. was kindly furnished by The Calco Chemical Company

these studies fails to inhibit the coagulation of rabbit blood, this holds true for the filtrate as well

In conclusion, the foregoing preliminary data indicate that the localizing property of *Staphylococcus aureus* is probably referable not merely to the severe irritating property of the organism *per se*, but also to its additional ability to release a powerful soluble exotoxin-like product, identical in many respects with leucocidin, and capable in itself of inducing a sufficiently intense injury to cause obstruction of normal lymphatic drainage. *Pneumococcus* Type I and *Streptococcus hemolyticus*, on the other hand, fail to form any such detectable accessory substance able to cause damage to the lymphatic or capillary endothelium. Briefly, then, evidences obtained thus far are in accord with the writer's original view, that the delayed fixation of dye and hence the invasive capacity of hemolytic streptococcus is referable to the mild local effects of this organism in contrast to the pronounced injurious action of *Staphylococcus aureus*. These studies are being continued in an attempt to obtain more precise information concerning the chemistry and the role of leucocidin-like substances

7599 P

### Anticoagulants of the Blood

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Any agent which inactivates or removes from blood, calcium, prothrombin, or thrombin is an anticoagulant. Theoretically, anti-fibrinogen and antithromboplastin are also possible but have never been convincingly demonstrated. The removal of calcium by oxalates, citrates, or fluorides is well known. As a type of antiprothrombin, aluminum hydroxide is an excellent example. On mixing and incubating oxalated plasma (0.5 cc) with aluminum hydroxide cream (0.05 cc) a plasma is obtained which after the removal of the aluminum hydroxide, will not clot on recalcification, whereas the untreated plasma clots in 2 minutes when calcium is added. The loss of clotting power is not due to removal of thromboplastin, for on adding an active preparation made from rabbit's brain, the normal clotting time is not restored. Furthermore, fibrinogen is not removed, for thrombin (fresh serum) will cause clot-

the immunity and cross-immunity was tested. The results follow.

**Immunity.** 8 rats immunized with venom were given venom in double the amount necessary to kill control animals. No symptoms appeared and all animals survived.

Three rats were given 8 A L D each of venom which had stood overnight mixed with 1 cc of serum from immunized rats. No symptoms appeared and all animals recovered. Venom treated with normal serum was not altered in potency.

Four rats were given 8 A L D each of venom followed immediately by 0.5 cc of serum from immunized rats. No symptoms appeared and all animals recovered.

Four rats were given 8 A L D of venom followed one hour later by 1 cc of serum. Symptoms had already appeared. Recovery was prompt and all animals recovered completely.

Four rats immunized against eggs were given 4 A L D of egg extract. No symptoms appeared. All survived.

Two rats were given 4 A L D of eggs after standing overnight mixed with 1 cc serum from egg-immunized rats. All survived without symptoms. Normal serum did not affect the potency of the egg poison, the rats dying within a few hours.

The above results represent the largest amounts of venom and egg poison used. Probably the potency of the serum is higher than indicated by these figures. This work is being continued.

**Cross-Immunization.** 3 rats given 2 A L D of venom which had stood overnight with serum from egg-immunized rats died within 6 hours.

Three rats given 2 A L D of eggs which had stood overnight mixed with serum-immunized rats died within 4 hours.

Immunity against eggs appeared more rapidly than against the venom. In the latter group little immunity could be demonstrated as the result of injections for the first month.

**Summary.** Anti-sera of considerable potency against both the venom and the eggs of *Latrodectus mactans* have been prepared. Cross-immunization experiments indicate that the 2 poisons are not identical.

## Anti-Serum Against Black Widow Spider Venom

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The most important, if not the only poisonous spider found in the United States is the so-called Black Widow (*Latrodectus mactans*), which is widely distributed and by no means uncommon. Some experimental work concerning the properties of the venom have appeared but to our knowledge no one has presented clear-cut evidence of having obtained anti-serum of high potency. There has also been considerable doubt as to whether the venom is different from the arachnolysin present in all spiders and which reaches its highest concentration in the eggs.

The authors have studied the black widow spider from the standpoint of its natural history and the chemical, physiological, pharmacological and immunological properties of its venom. Approximately one thousand spiders and several hundred rats have been used. The results of this investigation will be published elsewhere, we wish to report here only on the questions raised in the preceding paragraph.

Much of the previous work published was done on the effects of the spider bite. This is open to the criticism that the amount of venom introduced would vary with the size and anger of the spider and the amount and speed of absorption would vary with the depth to which the fangs had penetrated. Our method was to dissect the pair of venom glands from each of a large number of spiders (20 to 100 were used in each batch), and macerate the glands in saline. Injections were made intraperitoneally. A toxicity curve was plotted, 10 rats being used for each dosage, and the average lethal dose determined. The eggs were macerated in saline and the average lethal dose determined. We found that one-fourth the venom in one spider would kill 5 rats out of 10, while one-half of the venom would kill 9 out of 10. For the eggs, one egg would kill 4 out of 10, while 2 eggs would kill 10 of 10 rats. For the venom, therefore, one-fourth spider is considered the average lethal dose, and for the eggs, one and one-fourth eggs is considered the average lethal dose.

A group of rats was injected every other day for 2 months with sub-lethal amounts of venom and another group with sub-lethal amounts of eggs for the same period. At the end of that time

allowed to stand, with frequent stirring, in an incubator at 40°C for 48 hours. The liquid was then filtered off, the yield being about 1850 cc. This solution was then evaporated on a steam bath to about 250 cc, filtered, and the volume made up to 500 cc. A portion of this solution was then adjusted to pH 7.1 and allowed to set for 30 minutes in the ice box. A slight precipitate was observed. Filtration was made through a Seitz filter, while still cold, which removed the precipitate and rendered the solution sterile. This extract was then kept in the ice box until ready to be used.

In the data reported each growth determination was made in duplicate. Inoculations were made with 18-hour cultures of *Escherichia coli* or *Alcaligenes fecalis*. In every case 0.2 cc of a 0.1% broth suspension was used. In addition to the various amounts of solutions reported in the tables, 5.0 cc of infusion medium from the same batch were added to each tube, making the total volume in each case 12.2 cc. Growth was determined by centrifuging 10.0 cc of each culture in a Hopkins tube for 1 hour at a standard speed, and recording the growth in cubic centimeters. All tubes, including the control tubes for the original pH, were incubated together at 37°C for 8 hours. The hydrogen ion concentrations were determined with the hydrogen electrode. The buffer employed was McIlvaine's  $\text{Na}_2\text{HPO}_4$ —Citric acid<sup>2</sup> of pH 7.0. The glucose solution was prepared so that 1.0 cc. gave a 1% solution. The saline used was a 0.9% sodium chloride solution.

From the results of the extract in various dilutions upon the growth of *Escherichia coli* in unbuffered media (Table I) it will be

TABLE I.  
Effect of Extract on *E. coli* in Unbuffered Medium.

Extract cc.	Saline cc	Growth (cc.)			Gas	pH	
		1st tube	2nd tube	Aver		Original	Final
0.0	7.0	0.07	0.07	0.07	—	7.18	6.84
0.1	6.9	0.12	0.13	0.12	+	7.19	6.09
0.5	6.5	0.16	0.17	0.16	+	7.17	4.92
1.0	6.0	0.23	0.22	0.22	+	7.16	4.82

observed that there is a steady increase in growth with increasing amounts of the extract, but that the maximum stimulation has not been reached with the amounts of extract used. On the other hand, with varying amounts of glucose, under like conditions, (Table II) there is a constant stimulation which does not increase with increasing amounts of glucose. The decrease in pH in the latter case

<sup>2</sup> Clark, W. M., "The determination of hydrogen ions" Williams and Wilkins, Baltimore, Md. 1928 p. 214.

## Carbohydrate Nature of Pantothenic Acid (Williams)

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Williams *et al*<sup>1</sup> found that extracts from diverse tissues, representing different biological groups, contained material which had a very striking stimulation on the growth of Gebrude Mayer yeast. From a similarity of biological and chemical reactions it was concluded that this stimulation was due to a single substance, which they called pantothenic acid, and which appeared "to have a molecular weight of about 150, to be distinctly acid without amphoteric properties, to have several hydroxyl groups in its structure, but no olefin double bond, aldehyde, ketone, sulfhydryl, basic nitrogen, aromatic or sugar groups"

Following the discovery of this acid, experiments were conducted in this laboratory to determine the effect, if any, of this substance upon bacterial growth. The organism which seemed most suitable for this work was *Escherichia coli*, since it was readily cultured and possessed considerable carbohydrate fermentative ability. Rice bran was employed because it was readily obtained and conveniently extracted.

Preliminary experiments with extracts of rice bran which were prepared by the method of Williams, refluxing the bran with a 60% methyl alcohol solution, etc., showed considerable stimulation of the organism with small amounts of the extract, but much less stimulation than observed by Williams with yeast. The results in many cases were inconsistent due principally to the low pH of the extract and to the variations of the inoculum.

To overcome these inconsistencies and to standardize the procedure all the extracts were first adjusted to a pH between 6.9 and 7.3. Inoculations were made with a constant amount of a broth suspension of the organisms, generally 0.2 cc. No means were attempted to standardize the infusion media, no 2 batches of which were the same.

In later experiments a different procedure was used for the extraction of the rice bran. This procedure, which was used for the experiment to be reported, was as follows: 800 grams of rice bran were mixed with 3 liters of 25% aqueous ethyl alcohol solution and

<sup>1</sup> Williams, R. J., Lyman, C. M., Goodvear, G. H., Truesdail, J. H., and Holaday, D. J. *Am Chem Soc*, 1933, 55, 2912

TABLE V  
Effect of Extract on *E. coli* in Presence of 0.5% Glucose

Extract cc.	Glucose cc	Saline cc	Growth (cc)			Gas	pH	
			1st tube	2nd tube	Aver		Original	Final
0.0	0.5	6.5	0.13	0.13	0.13	+	7.18	4.74
0.1	0.5	6.4	0.13	0.13	0.13	+	7.19	4.77
0.5	0.5	6.0	0.16	0.18	0.17	+	7.17	4.79
1.0	0.5	5.5	0.20	0.20	0.20	+	7.16	4.85

TABLE VI  
Effect of Extract on *E. coli* in Presence of 1.0% Glucose

Extract cc	Glucose cc	Saline cc	Growth (cc)			Gas	pH	
			1st tube	2nd tube	Aver		Original	Final
0.0	1.0	6.0	0.12	0.13	0.12	+	7.18	4.69
0.1	1.0	5.9	0.12	0.13	0.12	+	7.19	4.71
0.5	1.0	5.5	0.16	0.17	0.16	+	7.17	4.76
1.0	1.0	5.0	0.23	0.23	0.23	+	7.16	4.85

increasing amounts of the extract, but on the other hand, increases. The probable explanation of this is that the extract itself contains some buffering salts.

Gas was produced during growth in all cases except with the 0.1 cc dilution of the extract in buffered medium (Table III) and the 0.1 cc dilution of glucose under the same conditions (Table IV). In these cases it is probable that the carbonic acid formed was less than the amount required to neutralize the buffer, and with no excess, no gas bubbles were observed to rise and collect at the surface.

To show this more conclusively, another experiment was performed (Table VII). Five cc of infusion medium were added to

TABLE VII  
The Final pH with Various Amounts of Extract and Buffer. Gas was produced in all tubes except those marked \*

Extract cc	Buffer			
	2.0 cc	4.0 cc	6.0 cc	8.0 cc
0.1	6.28	6.40*	6.61*	6.69*
0.5	5.45	5.75	6.02	6.19
1.0	5.25	5.52	5.89	6.08
2.0	5.29	5.58	5.78	5.97

each tube as listed in Table VII, and the total volume made up to 15.0 cc with saline. Inoculations were the same as in other experiments. Incubation time was 6 hours. These data show that with smaller amounts of the buffer, gas was observed in all cases where the final pH was less than 6.3, which coincides with the data shown in Tables III and IV.

is much greater than in the former, and is probably the limiting factor of the growth stimulation

TABLE II  
Effect of Glucose on *E. coli* in Unbuffered Medium

Glucose cc	Saline cc	Growth (cc)			Gas	pH	
		1st tube	2nd tube	Aver		Original	Final
0.0	7.0	0.07	0.07	0.07	—	7.18	6.64
0.1	6.9	0.13	0.13	0.13	+	7.18	5.71
0.3	6.7	0.12	0.13	0.12	+	7.18	4.78
0.5	6.5	0.13	0.13	0.13	+	7.18	4.74
1.0	6.0	0.12	0.13	0.12	+	7.18	4.69

TABLE III  
Effect of Extract on *E. coli* in Buffered Medium

Extract cc	Buffer cc	Saline cc	Growth (cc)			Gas	pH	
			1st tube	2nd tube	Aver		Original	Final
0.0	6.0	1.0	0.10	0.08	0.09	—	6.88	6.83
0.1	6.0	0.9	0.16	0.18	0.17	—	6.87	6.70
0.5	6.0	0.5	0.46	0.48	0.47	+	6.88	6.17
1.0	6.0	0.0	0.58	0.58	0.58	+	6.88	5.69

TABLE IV  
Effect of Glucose on *E. coli* in Buffered Medium

Glucose cc	Buffer cc	Saline cc	Growth (cc)			Gas	pH	
			1st tube	2nd tube	Aver		Original	Final
0.0	6.0	1.0	0.10	0.08	0.09	—	6.88	6.83
0.1	6.0	0.9	0.22	0.22	0.22	—	6.87	6.63
0.3	6.0	0.7	0.46	0.44	0.45	+	6.86	6.15
0.5	6.0	0.5	0.45	0.47	0.46	+	6.87	5.62
1.0	6.0	0.0	0.47	0.48	0.47	+	6.86	5.46

This is borne out more strikingly when buffered media were used (Tables III and IV), for with the extract much greater stimulation was recorded than in the unbuffered media, as was also true with glucose. However, the stimulation due to the glucose reaches a maximum, while that due to the extract does not. It is also observed that the stimulation produced by the extract is greater than that produced by the glucose and that there is a correspondingly smaller decrease in the pH with the former than with the latter, as compared with the growth increase.

When glucose is added together with the extract (Tables V and VI) there is no cumulative stimulation as would be expected if the stimulating agent were similar to glucose, nor is there a constant and unchanging stimulation as was observed with glucose alone (Table II). It will be noted that the final pH does not decrease with



with 100 gm Lloyd's reagent. The extractives were removed from the Lloyd's with barium hydroxide. The barium was removed from the filtrate with sulphuric acid. The filtrate containing creatine, creatinine and substances giving a positive Sakaguchi reaction was concentrated *in vacuo* to a volume of approximately 300 ml. This concentrated extract was kept in the refrigerator until there had accumulated a series of extracts from a total of 14.8 liters of urine representing a collection period of 18 days. These concentrates were then combined and evaporated to a volume of 1400 ml and treated in the usual manner with phosphotungstic acid. Sixty per cent of the substances responsible for the positive Sakaguchi reaction remain in the phosphotungstic acid filtrate. The filtrate is freed of phosphotungstic acid with barium hydroxide and the excess barium removed with sulfuric acid. The filtrate is evaporated almost to dryness and extracted with absolute alcohol. The residue insoluble in alcohol was dissolved in water and sufficient sulphuric acid added to give a 2% concentration. This solution was autoclaved at 15 pounds pressure for 30 minutes and was then again treated with phosphotungstic acid. The filtrate was evaporated to 25 cc. The addition of picric acid to this concentrated filtrate resulted in the formation of a picrate which was recrystallized once from water. Six hundred and sixty mg of the picrate was obtained which retained its crystalline form after drying at 100°C for several hours. The picrate melted with decomposition at 200.6° and showed no depression of melting point when mixed with glycocyamine picrate. This picrate gave a positive Sakaguchi reaction and gave the same amount of color as glycocyamine picrate when equal quantities were compared.

Three hundred and fifty mg of the picrate was suspended in 10 ml of 20% sulfuric acid and extracted with benzene. The sulphuric acid was removed with barium hydroxide and the solution evaporated to a volume of 5 ml, from which, on cooling, crystals were obtained in the form of rhomboid plates. These were recrystallized once from water and dried at 90°C. Dr S. A. Thayer of St. Louis University analyzed these crystals and obtained the following results:

3.813 mg gave 2.02 mg $H_2O$ and 4.312 mg $CO_2$		
Glycocyamine, $C_8H_7O_2N_3$ (117)	calculated C 30.77	H 5.98
	found C 30.83	H 5.93
1.890 mg gave 0.632 cc N at 30° C, B.P. 73.4 at 0°		
	calculated N 35.87	
	found N 35.68	

In another case of muscular dystrophy (12-year-old boy) receiv-

The effect of the extract upon *Alcaligenes fecalis* is entirely negative. This organism is known to have no carbohydrate-fermenting ability. As would be expected, the pH increases rather than decreases.

From the facts that the extract causes a decrease in the final pH, the production of gas, and has no stimulating effect on the growth of *Alcaligenes fecalis*, the assumption can be made that the stimulating agent, probably pantothenic acid, is related to the carbohydrates. The stimulation observed with the extract, however, can in no way be attributed to glucose or other hexoses which stimulate *E. coli* in a manner similar to glucose.

**Conclusion** 1 Rice bran contains a growth-stimulating agent for *Escherichia coli*, but not for *Alcaligenes fecalis*. 2 This stimulating agent is probably related to the sugars. 3 It is not a hexose. 4 The substance is probably identical with the pantothenic acid of Williams.

## 7602

### Isolation of Glycocyamine from Urine

C J WEBER (Introduced by E A DOISY)

*From the Department of Internal Medicine, University of Kansas School of Medicine*

The isolation of glycocyamine (guanido acetic acid) from urine is of interest because of its possible relationship to the origin of creatine. Hunter has critically reviewed the literature on this subject in his monograph on "Creatine and Creatinine".

My interest in this subject began with the finding that an extract of urine prepared with Lloyd's reagent gave a positive Sakaguchi reaction. I also found that the Lloyd's extract of urine from a case of pseudohypertrophic muscular dystrophy gave a stronger Sakaguchi reaction than did urine from normal individuals. Urine from this patient (8-year-old boy) was therefore used in attempting to isolate the substance responsible for the positive reaction. The patient received 15 gm of glycine daily during the period of urine collection. The urine was collected in 2- or 3-day periods using toluene as a preservative.

The procedure used is briefly as follows. The urine after filtering was made acid to congo red with hydrochloric acid and extracted

main the previous results and have extended the experiments to follow the respiratory changes in *P. polycephalum*. As gauze sieves I have used Latimer's "Old Anchor Brand" calibrated bolting cloth Nos 00, 4, 6, 8, 10, 14, and 21 which have average pore sizes of 0.75, 0.32, 0.24, 0.20, 0.155, 0.10 and 0.07 respectively. When strained through the last 3 the plasmodium gave no growth, when the 0.20 sieve was used the strained plasmodium sometimes grew, sometimes not, with sieves of larger pores active plasmodium when forced through, grew on the culture medium. On the other hand, plasmodium if allowed to grow in gauze bags, passed through all the sieves as well as hard paper filters onto the culture medium. In 5 hours plasmodium made its way through hard filter S & S No 575A which has an average pore size of  $1\ \mu$ . These facts confirm the conclusions previously stated that essential to the life of the plasmodium are filaments or fibrils which may be 0.20 mm in length but in diameter are below visibility with the microscope.

Since viability is so conspicuously reduced by comminuting the plasmodium it seemed important to determine whether metabolism as indicated by consumption of oxygen might not also be modified in this way. The determinations were carried out in the usual way with the Warburg apparatus. A quantity of plasmodium, approximately 20 mg, was spread in a thin layer on a piece of platinum foil and introduced into the respiration chamber which was then charged with oxygen. The temperature of the bath was kept at  $21^{\circ}\text{C}$ . One or 2 normal controls were run with the experimental charges. Half hourly readings were taken over a period of 3 hours. The samples were then removed, dried to constant weight over soda lime, and weighed to the nearest 0.1 mg. The experiments all showed that  $\text{O}_2$  consumption was reduced as a result of forcing the plasmodium through sieves, and that the  $\text{O}_2$  consumed decreased with lessening of the pore size of the sieve used. The average value of  $\text{O}_2$  consumption for normal plasmodium (36 readings) was found to be 4.3 cu mm per mg dry weight per hour. For material passed through 0.75 mm pores the  $\text{O}_2$  consumption was 73% of normal, while through 0.07 mm pores the figure was 49% of normal. Thus a degree of comminution which always killed the plasmodium reduced the consumption of  $\text{O}_2$  by about 50%. These facts suggest that structures in the cytoplasm of *P. polycephalum* are essential both to its life and to the greater part of its respiratory activity.

It was further found that deformation of the plasmodium without comminution caused a delay in growth and a reduction in  $\text{O}_2$

ing 15 gm of glycine daily, 274 mg of glycoyamine picrate was isolated from the urine over an 8-day period. In this case the urine was collected daily and extracted with Lloyd's reagent on the same day. The quantity of glycoyamine picrate isolated represents 23% of the original Sakaguchi reacting substances in the Lloyd's extract using glycoyamine as the standard for comparison.

In a control experiment 600 mg of glycoyamine was added to a solution containing creatin, creatinine, glycine, urea and salts in concentrations approximating that found in the above 8-day urine collection. This mixture was treated by exactly the same procedure used above. Three hundred and ninety-six mg of glycoyamine picrate was isolated which represents a recovery of approximately 33% of the glycoyamine extracted by Lloyd's reagent as determined by the Sakaguchi reaction. This suggests that probably the major part if not all of the Sakaguchi reacting substance in the Lloyd's extract from urine is glycoyamine.

Glycoyamine is not produced during the process of isolation, at least, from creatine, creatinine or glycine and urea. The addition of creatine, creatinine, glycine or urea together, or separately, to urine causes no increase in the Sakaguchi reaction of the Lloyd's extract.

The feeding of glycine to a patient with pseudohypertrophic muscular dystrophy results in an increase of approximately 60% in the Sakaguchi reacting substances in the urine.

### 7603 P

#### Relation of Cytoplasmic Structure to Growth and Respiration in Plasmodium.

A. R. MOORE

*From the Effingham B. Morris Biological Farm of the Wistar Institute of Anatomy and Biology*

Recently I have shown that if a small piece of the plasmodium of *Physarum polycephalum* be forced through a gauze with sufficiently small pores (less than 0.25 mm in diameter) subsequent growth of this material does not occur, whereas if the pores are larger, growth takes place when the material is put on the culture medium. The experiments were repeated and confirmed for *P. rigidum*. During the present summer I have confirmed in the

solution or tap water were added alternately to the returned secretions. In addition to the fluids mentioned 500 to 1500 cc of Ringer's solution were given subcutaneously daily to prevent dehydration. The control period lasted from 3 to 7 days. After the animal had fully recovered from the operation and the volume of the secretions had reached a constant level a second operation was performed. The jejunum was divided and the ends inverted just proximal to the jejunal cannula, producing an isolated duodeno-jejunal loop 15 to 20 cm long around Treitz' ligament. Following this the collections and replacements were made in the usual manner. When replacement of the secretions was impossible larger amounts of Ringer's solution were given subcutaneously. In the obstructive period vomiting occurred. The volume of vomitus was prorated and added to the amounts of secretion collected. In 6 of the 10 animals the results were complicated by the following occurrences: gastric bleeding, perforation of the loop without distention, leakage around the cannula, failure of the secretions to assume a stable level during the control period, and internal hernia obstruction. In 5 of these animals the secretions were diminished in volume after the production of the isolated loop. In the animal with the bleeding into the stomach and duodenum the secretions were increased.

In the remaining 4 dogs the results were free from complications. Two of these were operated on under ether, and 2 under 2% novocaine anesthesia. Only one of the latter 2 had morphine prior to operation. In the control period the secretions reached a level in from one to 2 days averaging from 50 to 70 cc per hour. Following the obstruction these animals survived for from 2 to 4 days during which time the secretions never exceeded 45 cc per hour. In each individual instance the volume of the digestive secretions after the production of the isolated loop was definitely less than the volume obtained during the control period. At autopsy these 4 animals showed a distended gangrenous loop without gross perforation.

It was thought that the second operation might have a depressant influence upon the digestive organs. Therefore, in a second group of 12 dogs an effort was made to produce an isolated loop without the second operation. Various methods tried on 8 animals failed. Then in 4 dogs the duodenal and jejunal cannulae were placed as in the first group and an isolated duodeno-jejunal loop was made with a round flanged brass cannula placed by means of 2 purse string sutures into the distal end to drain the loop. During the control

consumption The deformation was accomplished by centrifuging the samples with an air driven centrifuge (H W Morse's model of the Henri-Huguenard centrifuge) at approximately 75,000  $\times$  gravity for from 1 to 10 minutes Centrifuging for 6 minutes caused a delay of about 12 hours in subsequent growth of the piece, and an immediate reduction in the consumption of oxygen to near 50% These results indicate that growth and respiration depend upon an intimate association of elements of relatively low and high specific gravity in the plasmodium, that these can be separated by centrifuging to the impairment of growth and respiration But unlike comminution, the effects of separation by centrifuging are reversible

## 7604 P

## Influence of Closed Intestinal Loop Strangulation on Volume of the Combined Digestive Secretions.\*

JOSEPH M SWINDT AND M LAURENCE MONTGOMERY (Introduced by C D Leake)

*From the Department of Surgery, University of California Medical School, San Francisco*

We have reported our findings<sup>1</sup> on the influence of simple duodenal obstruction upon the volume of the combined gastric, biliary, pancreatic, and duodenal secretions In the present work we have studied the influence of closed intestinal loop strangulation upon these same secretions Twenty-two fasting healthy dogs were used in the study All had their salivary ducts ligated

In the first group of animals the duodenum was divided below the entrance of the lower pancreatic duct, a Dragstedt type of intestinal cannula was placed in the proximal duodenum and the cut ends were inverted A second cannula was placed in the jejunum about 6 to 8 cm distal to Treitz' ligament From the duodenal cannula the combined digestive secretions drained into a clean rubber bag Collections were made every one to 4 hours day and night, measured, and returned by cannula into the jejunum About 50 cc of Ringer's

\*This work was conducted under a grant from the Christine Breon Fund for Medical Research of the University of California Medical School

<sup>1</sup> Montgomery, M L, and Swindt, J M, *Proc Soc Exp Biol and Med*, 1934, **31**, 915

duced death. In embolism due to introduction of air into a peripheral vein, the air goes first to the heart and lungs independent of the position of the animal. In the present studies, all animals were kept flat in the supine position during the injection of gas. The gas was injected quite rapidly, in most instances the entire amount being introduced in about 30 seconds.

The minimal fatal dose of air was found to be about 8 cc per kilo body weight. Animals under urethane anesthesia were used throughout the work. The air was injected through a large cannula into the femoral vein (usually the left) from an inverted burette, being forced in by a column of water. Another cannula was placed in the carotid artery to record the blood pressure. The air flowed in rapidly until at a certain point it began to flow more slowly and the column of water showed pulsations synchronous with the heart beat. This usually occurred when very roughly nine-tenths of the minimal fatal dose was injected. The results of 11 air injections are shown in Table 1.

TABLE I  
Effects of Injection of Air into the Femoral Vein

No	Dog Wt Kg	Cc air injected	Cc per Kg	Time of injection sec	Result
1	6.5	150	23.1	960	death
2	7.0	55	7.9	205	"
3	9.0	45	5.0	7	"
4	9.5	47	5.0	75	recovery
5a	8.0	54	6.8	10	"
5b	8.0	64	8.0	10	death
6	8.5	68	8.0	22	"
7	12.0	68	5.7	38	recovery
8	12.0	72	6.0	32	death
9	6.9	64	9.3	16	"
10	7.0	56	8.0	18	"
11	19.0	178	9.4	40	"

A dog that recovered following an injection of air or oxygen usually showed a rapid fall in blood pressure to as low as 20 mm of mercury with gradual recovery to the normal level. There was usually no diminution in pulse pressure, but some slowing of the heart rate. Necropsy shortly after recovery in one dog showed no air or oxygen in the heart. Recovery was usually complete within 2 or 3 minutes.

A dog that died following an injection of air or oxygen usually showed a rapid fall in blood pressure to about 16 mm of mercury with no return to the normal level. There was a rapid decrease in the pulse pressure with some slowing of the heart rate. The pulse

period the collections and replacements of the digestive secretions were made in the usual manner. Then the isolated loop was closed by plugging the drainage cannula with cotton and screwing a cap on the end. In 2 of these animals there was leakage around the cannula which allowed the loop to decompress and prevented strangulation. However, in 2 animals the closure was perfect. The loops became distended, necrotic, and perforated as revealed at autopsy. During the control period the secretions in these 2 animals averaged about 65 cc per hour whereas the highest value after obstruction of the loop was 50 cc per hour. Often it was much lower. One animal showed a sharp terminal rise in secretion, which, however, did not exceed the average for the control period. The character and amount of the immediately preceding secretions indicated this probably was due to terminal relaxation of the pylorus and emptying of accumulated fluid in the stomach rather than to stimulation. In the second dog the stomach was aspirated frequently during the period of obstruction and the terminal rise did not occur. It is our belief, therefore, that in isolated duodeno-jejunal loop strangulation obstruction the combined gastric, pancreatic, biliary and upper duodenal secretions are not stimulated to excessive secretion. On the contrary they tend to be depressed.

## 7605

## Embolism by Air and Oxygen Comparative Studies \*

HENRY N. HARKINS AND PAUL H. HARMON (Introduced by Edmund Andrews)

*From the Department of Surgery, The University of Chicago*

Attempts were made in these experiments to compare the minimal fatal dose of air and oxygen when introduced into a peripheral vein. Van Allen, Hrdina and Clarke<sup>1</sup> found that in embolism due to air introduced into the pulmonary vein, the rapidity of introduction of the air and the position of the animal were 2 factors of prime importance. The position of the animal was believed to be of importance by directing the flow of air upward by gravity. These authors found that when the head was uppermost, the air more easily pro-

\* Work done in part under a grant from the Douglas Smith Foundation. Preliminary report

<sup>1</sup> Van Allen, C. M., Hrdina, L. S., and Clarke, J., *Arch. Surg.* 1929 **19**, 567



duced death. In embolism due to introduction of air into a peripheral vein, the air goes first to the heart and lungs independent of the position of the animal. In the present studies, all animals were kept flat in the supine position during the injection of gas. The gas was injected quite rapidly, in most instances the entire amount being introduced in about 30 seconds.

The minimal fatal dose of air was found to be about 8 cc per kilo body weight. Animals under urethane anesthesia were used throughout the work. The air was injected through a large cannula into the femoral vein (usually the left) from an inverted burette, being forced in by a column of water. Another cannula was placed in the carotid artery to record the blood pressure. The air flowed in rapidly until at a certain point it began to flow more slowly and the column of water showed pulsations synchronous with the heart beat. This usually occurred when very roughly nine-tenths of the minimal fatal dose was injected. The results of 11 air injections are shown in Table 1.

TABLE I  
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5a	8.0	54	6.8	10	"
5b	8.0	64	8.0	10	death
6	8.5	68	8.0	22	"
7	12.0	68	5.7	38	recovery
8	12.0	72	6.0	32	death
9	6.9	64	9.3	16	"
10	7.0	56	8.0	18	"
11	19.0	178	9.4	40	"

A dog that recovered following an injection of air or oxygen usually showed a rapid fall in blood pressure to as low as 20 mm of mercury with gradual recovery to the normal level. There was usually no diminution in pulse pressure, but some slowing of the heart rate. Necropsy shortly after recovery in one dog showed no air or oxygen in the heart. Recovery was usually complete within 2 or 3 minutes.

A dog that died following an injection of air or oxygen usually showed a rapid fall in blood pressure to about 16 mm of mercury with no return to the normal level. There was a rapid decrease in the pulse pressure with some slowing of the heart rate. The pulse

became imperceptible to palpation and on the blood pressure tracing. However, as long as 3 minutes after the last visible cardiac pulsations, a few abortive respirations would occur. In some cases a few weak cardiac pulsations occurred as long as 8 minutes after death as seen when the chest was opened. Necropsy showed air or oxygen in the vena cava and right side of the heart.

Oxygen injections were only slightly less toxic than air injections as seen in Table II. All effects on the blood pressure, respiration, and pulse were identical.

TABLE II  
Effects of Injection of Oxygen into the Femoral Vein

No	Dog Wt kg	Cc air injected	Cc per kg	Time of injection sec	Result
12a	7.8	62	8.0	20	recovery
12b	7.8	62	8.0	22	"
12c	7.8	69	8.9	26	"
12d	7.8	78	10.0	37	"
13a	9.5	65	6.8	30	"
13b	9.5	76	8.0	25	death
14	9.5	75	7.9	35	"
15	8.0	72	9.0	38	"
16	6.5	72	11.0	28	"
17	7.5	75	10.0	33	"
18a	8.0	80	10.0	30	recovery
18b	8.0	100	12.5	22	death

In an 8 kg dog there is about 615 cc of blood (assuming one-thirteenth of the body weight is blood). The arterial blood may be considered to be completely saturated with oxygen. Then assuming that half of the blood in the body is venous and that this half is 6% unsaturated with oxygen (it is realized that the unsaturation depends on vasomotor conditions and varies in various parts of the body), then the blood of the entire body of an 8 kg dog is  $0.06 \times 308 = 19$  cc unsaturated. Since air is about 20% oxygen, and the minimal fatal dose of air for such a dog is 64 cc, this amount of air would contain 13 cc oxygen. Thus the air would be taken up by the hemoglobin to the extent of 13 cc while 19 cc of oxygen might be taken up. Theoretically therefore, from the standpoint of combining with hemoglobin, the 2 should act practically the same. It is quite possible that because of the rapidity of the injection, the oxygen or air does not have time to mix with all the venous blood. Except for this it might be expected that an 8 kg dog could tolerate 19 cc more air or oxygen than pure nitrogen.

An attempt was made in experiments 13b and 15 to increase the oxygen unsaturation by clamping off the trachea for 60 and 75

seconds respectively just before injecting the oxygen. It is seen that the oxygen tolerance was not increased by this procedure. No blood studies were made in these dogs.

Calculations are made that indicate that the minimal fatal dose of oxygen should not be greater than that of air when injected rapidly into the peripheral vein of a dog. The results of 24 injections of air or oxygen into 18 dogs show no marked difference in toxicity.

## 7606 C

## Studies on Centrifuged Frog Eggs

H. W. BEAMS, R. L. KING, AND P. L. RISLEY (Introduced by E. Witschl.)

*From the Zoology Laboratory, State University of Iowa*

The effect of centrifuging fertilized eggs of the frog both before cleavage and during the early cleavage stages has been studied by several investigators. Gurwitsch,<sup>1</sup> Hertwig,<sup>2</sup> Wetzel,<sup>3</sup> Morgan,<sup>4</sup> Konopocka,<sup>5</sup> McClendon,<sup>6</sup> and Jenkinson<sup>7</sup> particularly have reported the redistribution of certain materials and the intense stratification of others along the primary axis of centrifuged frog eggs. They state that the stratification of materials takes place in the order of their relative specific gravity, i. e., a yellow or white centripetal layer, a translucent protoplasmic middle layer and a heavy yolk centrifugal layer containing the black pigment granules. In general, the results of these investigators show that, when the centrifuging has been relatively weak, development is often apparently normal with perhaps a slight abnormal pigmentation and distribution of fat in the head region. However, if the centrifugal force has been slightly greater, curious monsters often appear. Jenkinson particularly has noted that the myotomes and spinal ganglia are frequently fused together beneath the nerve tube and that the notochord is often absent altogether. Hertwig frequently obtained monstrosities of the spina bifida type. Pasquini and Reverberi<sup>8</sup> have centri-

<sup>1</sup> Gurwitsch, A., *Verhandl. Anat. Gesellsch.*, 1904, **18**, 146

<sup>2</sup> Hertwig, O., *Arch. f. Mikr. Anat.*, 1904, **63**, 643

<sup>3</sup> Wetzel, G., *Arch. f. Mikr. Anat.*, 1904, **63**, 636

<sup>4</sup> Morgan, T. H., *Arch. f. Entw. Mech.*, 1906, **22**, 553

<sup>5</sup> Konopocka, B., *Bull. Int. Acad. Crac.*, 1909, Ser. B, 689

<sup>6</sup> McClendon, J. F., *Arch. f. Entw. Mech.*, 1909, **27**, 247

<sup>7</sup> Jenkinson, J. W., *Quart. J. Micr. Sci.*, 1915, **60**, 61

<sup>8</sup> Pasquini, P., and Reverberi, G., *Boll. Inst. di Zool.*, Univ. Roma, 1929, **7**, 1

fused frog eggs at the gastrula stage and obtained many curious monsters not unlike some that have appeared in our experiments

We have recently centrifuged unfertilized frog eggs of *Rana aurora* for 2 minutes at 40 pounds pressure in the air-driven ultracentrifuge described by Beams, Weed and Pickles<sup>9</sup> It has been possible to repeat the stratification of materials in the frog egg as described by the above named investigators Furthermore, we have produced a complete cleavage or fragmentation of the egg into a light fatty fragment and a heavier yolk and protoplasmic fragment No effort has been made to determine whether or not such fragments may be fertilized and if fertilized will develop When stronger centrifugal force is applied the fragments are completely forced out of the jelly and burst

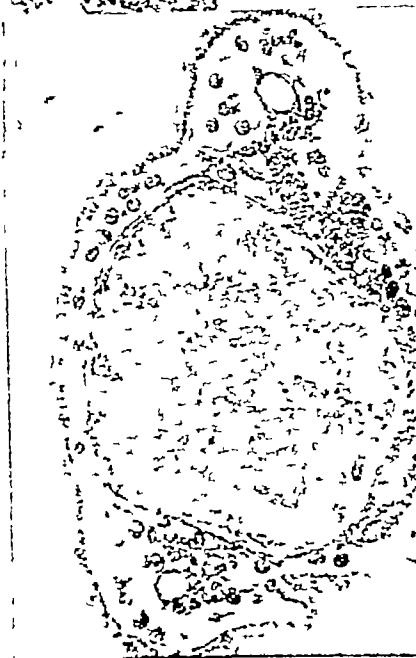
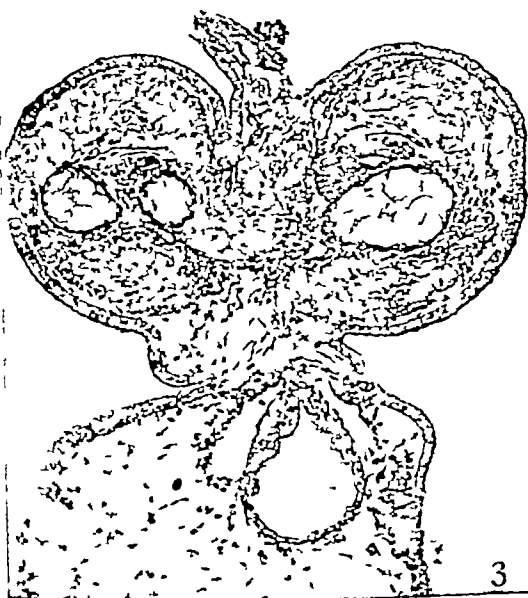
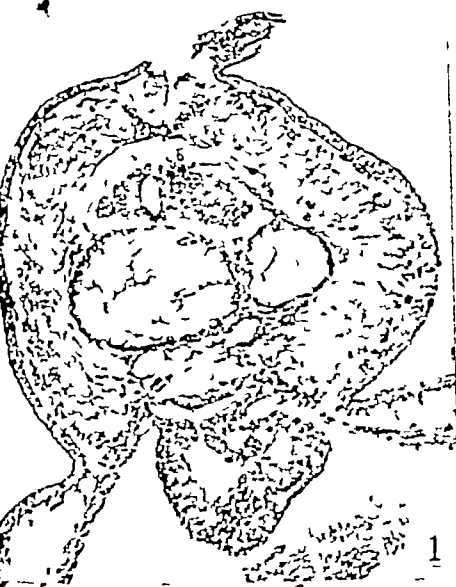
In another series of experiments the eggs of *Rana pipiens* in the many cell and early gastrula stages were centrifuged for 5 to 7 minutes at about 2000 times gravity Such eggs often develop into monsters, many of which possess 2 and sometimes 3 tails Figure 1 is a cross section through the region of the base of the tail of such a tadpole It will be noted that the neural tube is single but shows some indication of spreading and becoming divided as in Figures 2 and 3 which are serial sections of the same tadpole taken slightly more caudad The notochord has already doubled in Figure 1, and in Figures 2 and 3 the right notochord has divided so that there are 3 distinct notochords with 2 neural tubes Sections taken cephalad show the neural tube and notochord to be single

Figure 4 represents a cross section of another tadpole of a similarly treated group of eggs preserved at a slightly earlier period in development There are two distinct notochords present but no trace of a neural tube In this animal the brain showed distinct malformation and the neural tube extended posteriorly only for a very short distance Other monstrosities with no heads and expanded growths of epidermis into folds and ridges were found In still other cases the yolk was collected in the blastopore region forming an unusually large and persistent yolk plug which prevented the normal closure of the blastopore

No special effort was made to orient the eggs in the centrifuge and it is assumed that they arranged themselves in the usual way with the vegetal pole outward or centrifugally About one-third of the eggs which received the proper degree of centrifuging developed abnormally Controls developed normally in every case

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<sup>9</sup> Beams, J. W., Weed, A. J., and Pickles, E. G., *Science*, 1933, **78**, 338



We are unable to offer a definite explanation regarding the cause of the doubling of the neural tube and notochord and of the other curious malformations mentioned above. However, they may be due to mechanical disturbances in development caused by a displacement of mitotic figures, by the injury and possible killing of certain cells, or by the intense stratification of materials which results in an upset of the processes of development. On the other hand, it seems equally plausible from the work of Spemann and his collaborators that centrifuging in the many cell and early gastrula stages may have affected the composition or organization of the "inductors" or organizing centers resulting in a disturbance of the normal inductive process.

## 7607 C

Morphological Comparison of Anterior Pituitaries of Normal  
Castrated Female Rats and Those Receiving Injections  
of Pregnancy Urine Extracts \*

J. M. WOLFE

*From the Department of Anatomy, Vanderbilt University School of Medicine,  
Nashville, Tennessee*

It has been reported by Severinghaus<sup>1</sup> and Wolfe, Ellison and Rosenfeld<sup>2, 3</sup> that while injections of extracts of pregnancy urine result in marked changes in the anterior pituitaries of normal female rats, such extracts are without action on the anterior pituitaries of castrated female rats. In our previous reports<sup>2, 3</sup> detailed data were not given, in this report we wish to present quantitative data which demonstrate that the anterior pituitaries of non-injected castrated female rats (both mature and immature) are morphologically indistinguishable from those of female rats receiving injections of extracts of pregnancy urine.

Sixty-five virgin mature and 31 immature female rats were cas-

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\* These studies were supported by grants from the Committee for Research in Problems of Sex of the National Research Council, from the Committee for Scientific Research of the American Medical Association, and the Division of Medical Sciences of the Rockefeller Foundation.

<sup>1</sup> Severinghaus, A. E., *Proc Soc Exp Biol and Med*, 1934, **31**, 593

<sup>2</sup> Wolfe, J. M., Ellison, E. T., and Rosenfeld, Louis, *Anat Rec*, 1934, **58**, 93 (supplement)

<sup>3</sup> Wolfe, J. M., Ellison, E. T., and Rosenfeld, Louis, *Anat Rec*, 1934, **58**, 94 (supplement)

TABLE I

Period of cus- tration	Percentage of Cells	Frequency Distribution				Mean and Standard Deviation (Percent			
		Controls		Experimentals		Controls		Experimentals	
		Mature	Immature	Mature	Immature	Mature	Immature	Mature	Immature
15 day	Eosinophiles								
	30.0-34.9	2	2	4	2	Mean 36.5	Mean 38.4	Mean 37.5	Mean 38.3
	35.0-39.9	8	10	7	11	S D 2.0	S D 2.9	S D 3.6	S D 2.8
	40.0-44.9	—	3	4	3				
	Basophiles								
	50.0-59.9	1	—	2	—				
	60.0-69.9	3	—	6	—				
	70.0-79.9	4	—	7	—				
	80.0-89.9	2	1	—	—	Mean 9.4	Mean 16.0	Mean 8.7	Mean 10.1
	90.0-99.9	—	5	—	3	S D 1.8	S D 2.4	S D 1.3	S D 1.4
30 day	Eosinophiles								
	30.0-34.9	—	—	—	—				
	35.0-39.9	—	5	—	4				
	40.0-44.9	—	9	2	11	Mean 55.0	Mean 46.1	Mean 53.8	Mean 46.5
	45.0-49.9	5	1	8	1	S D 2.5	S D 2.9	S D 5.2	S D 2.6
	50.0-54.9	5	—	4	—				
	55.0-59.9	—	—	—	—				
	60.0-64.9	—	—	1	—				
	Chromophobes								
	35.0-39.9	—	—	—	—				
30 day	Eosinophiles								
	35.0-39.9	2	—	15	—	Mean 43.0	—	Mean 40.0	—
	40.0-44.9	6	—	9	—	S D 3.5		S D 3.2	
	45.0-49.9	5	—	2	—				
	Basophiles								
	50.0-59.9	1	—	3	—				
	60.0-64.9	—	—	10	—				
	65.0-69.9	6	—	11	—	Mean 14.9	—	Mean 12.9	—
	70.0-74.9	4	—	2	—	S D 2.01		S D 2.2	
	75.0-79.9	—	—	—	—				
30 day	Chromophobes								
	35.0-39.9	6	—	2	—	Mean 40.9	—	Mean 47.1	—
	40.0-44.9	5	—	4	—	S D 3.5		S D 4.2	
	45.0-49.9	3	—	14	—				
	50.0-54.9	—	—	6	—				

The quantitative data are arranged in statistical form. The frequency distribution, the mean and the standard deviation of the various groups are indicated.

trated The mature females were sacrificed at 15 and 30 day intervals after operation Forty-one of these rats received from 25 to 75 units of an extract of pregnancy urine<sup>†</sup> daily throughout the 15 day castration period or for the last 15 days of the 30 day castration period in those rats which were sacrificed after 30 days All immature rats were between 25 and 30 days old at operation, and were sacrificed 15 days after operation Sixteen received 25 units of pregnancy urine extract daily for the entire period, 15 littermate sisters served as controls

At autopsy the pituitaries were weighed and fixed in Regaud's fluid Serial sections of all glands were cut Complete cell counts were made on 5 sections from each of the 90 glands A total of 331,056 cells were counted

The quantitative results of these studies are presented statistically in Table 1 (Frequency distribution, means and standard deviations are given) Analysis of this table reveals that the percentages of the various cell types in the anterior pituitaries of the injected rats were almost identical to the percentages of these cells in the anterior pituitaries of the control rats castrated for a similar period of time Morphologically the anterior pituitaries of the injected rats and those of the controls appeared identical From the results of these experiments we feel justified in concluding that extracts of pregnancy urine are without action on the anterior pituitaries of castrated female rats

### 7608 C

#### Comparative Quantitative Effects of Castration in Mature and Immature Female Rats \*

J M WOLFE

*From the Department of Anatomy, Vanderbilt University School of Medicine, Nashville, Tenn*

Ellison and Wolfe<sup>1</sup> have reported that in the anterior pituitaries of castrated mature female rats there is an increase in the percentages

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<sup>†</sup> Follutein was furnished by E R Squibb and Sons through the courtesy of Dr J J Durrett.

\* These studies have been supported by the Committee for Research in Problems of Sex of the National Research Council, the Committee for Scientific Research of the American Medical Association, and the Division of Medical Sciences of the Rockefeller Foundation

<sup>1</sup> Ellison, E T, and Wolfe, J M, *Endocrinology*, 1934, 18, 555



TABLE I

Percentage of Cells	Mean Standard Deviation (Percent)			
	Frequency Distribution		30 day castrates	
	Mature	Immature	Mature	Immature
Leucocytes				
30 0 34 0	0	4	15	
40 0 44 0	4	—	7	
15 0 19 0	—	—	—	
Basophiles				
50 0 60 0	3	—	—	
70 0 80 0	9	—	1	
90 0 100 0	11	1	10	
110 0 120 0	2	8	17	
130 0 140 0	—	13	6	
150 0 160 0	—	8	2	
170 0 180 0	—	—	—	
190 0 200 0	—	1	—	
210 0 220 0	—	—	—	
Chromophobus				
150 0 160 0	—	9	8	
180 0 190 0	—	20	9	
200 0 210 0	2	—	16	
220 0 230 0	13	—	6	
240 0 250 0	9	—	—	
260 0 270 0	1	—	—	
280 0 290 0	—	—	—	
300 0 310 0	—	11	30	
No. of rats	25	11	30	

The frequency distribution, the mean and the standard deviation of the various groups are indicated

trated. The mature females were sacrificed at 15 and 30 day intervals after operation. Forty-one of these rats received from 25 to 75 units of an extract of pregnancy urine daily throughout the 15 day castration period or for the last 15 days of the 30 day castration period in those rats which were sacrificed after 30 days. All immature rats were between 25 and 30 days old at operation, and were sacrificed 15 days after operation. Sixteen received 25 units of pregnancy urine extract daily for the entire period, 15 littermate sisters served as controls.

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<sup>1</sup> Ellison, E. T., and Wolfe, J. M., *Endocrinology*, 1934, **18**, 555.

castrated for a period of time which was twice as great (30 days) Study of the frequency distribution of these cells in the mature and immature groups will show the individual variations in the different groups and demonstrate in a clear-cut fashion that the factor of age must be considered seriously when one is studying the reaction of the anterior pituitary of the female rat to castration. It is impossible at the present time to give an explanation of this interesting finding. It is pointed out that the initial levels of the basophiles are much higher in normal immature female rats than they are in mature females killed during the various phases of the oestral cycle. Also in the anterior pituitaries of immature female rats a great majority of the basophiles are completely filled with granules, while in the mature female these cells undergo cyclic changes in their granular content.

Reference to Table I will show that the mean level of eosinophiles in 39 female rats castrated for 30 days was 41.2% while the mean level of these cells in 69 mature normal female rats was 33.6%. This would indicate that in this group of castrates there was some increase in the eosinophiles. However, it is important to point out that occasionally the level of these cells in non-castrated females was slightly higher than 40%. Since the mean level of these cells in the 30-days castrates was only slightly above the upper limits of normal for normal females it seems questionable to conclude without additional data that castration in mature female rats results in an increase in the eosinophiles.

## 7609 C

### Suspension Stability of Erythrocytes in Solutions of Gum Acacia \*

S. P. LUCIA AND J. W. BROWN (Introduced by D. M. Greenberg)

*From the Division of Medicine, University of California Medical School, San Francisco, California.*

It is stated that the suspension stability of erythrocytes is dependent upon variations in the albumin, globulin, and fibrinogen content of plasma<sup>1, 2</sup>. An increase in the fibrinogen, or globulin, tends to diminish the stability<sup>3</sup>. A careful study of this phenome-

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\* Assisted by a grant from the Christine Breon Fund.

<sup>1</sup> Fahraeus, R., *Acta Med Scand*, 1921, **40**, Suppl.

<sup>2</sup> Westergren, A., Juhlin Dannfelt, C., and Schnell, R., *Acta Med Scand*, 1932, **77**, 469.

of the basophiles to a mean level of 9% in the 15-day castrates and 13.6% in the 30-day castrates. A few signet-ring castration cells were also found in the anterior pituitaries of the 30-day castrates. In further studies<sup>2</sup> we have pointed out that injection of pregnancy urine extracts<sup>7</sup> are without action on the pituitaries of mature female rats castrated for 15 and 30 days. By adding the 2 series of injected and uninjected animals together, we have to date studied and made cell counts on the anterior pituitaries of 25 mature females castrated for 15 days and 39 castrated for 30 days. These data are arranged in statistical form in Table I, in which the frequency distribution of the percentages of the various cell types, the mean and the standard deviation are given. In addition, 31 immature female rats were castrated when between 25 and 30 days of age and sacrificed 15 days later. Sixteen of these animals received injections of pregnancy urine extracts. Since these extracts were without action on the anterior pituitary,<sup>3</sup> data from these 31 rats are considered together and arranged statistically in Table I. For non-castrated controls we have to date made cell counts on the anterior pituitaries of 69 mature female rats killed at various periods of the oestral cycle, and 31 immature female rats killed between 25 and 35 days of age. The mean levels of the eosinophiles in the anterior pituitaries of the mature and immature controls were 33.6 and 36.1%, respectively. The standard deviations (S. D.) were 4.5 and 3.9, respectively. The mean level of the basophiles in the mature controls was 4.8% (S. D. of 1.2) and in the immature controls was 7.2% (S. D. of 1.3). The mean levels of the chromophobes were 61.9% (S. D. of 4.2) in the mature controls and 55% (S. D. of 3.2) in the immature controls.

Analysis of Table I reveals that in 25 mature female rats castrated for 15 days, the mean level of the basophiles was increased to 9% and in 39 mature females castrated for 30 days, the mean level of the cells was increased to 13.6%. (The mean level in 69 non-castrated mature females was 4.8%). Further analysis reveals that in 31 immature rats, castrated before they were 30 days of age and sacrificed 15 days after removal of the ovaries, the mean level of the basophiles was increased to 15.8%, a mean percentage which was slightly higher than that found in mature female rats.

<sup>2</sup> Wolfe, J. M., Ellison, E. T., and Rosenfeld, Louis, *Anat. Rec.*, 1934, suppl. ment, 58-94.

<sup>7</sup> Follutein was furnished by E. R. Squibb and Sons through the courtesy of Dr. J. J. Durrett.

<sup>3</sup> Wolfe, J. M., *Proc. Soc. Exp. Biol. and Med.*, 1934, 32, 184.

Locke's solution, 1, 2, 3, 4, 6, 8 and 10% acacia in buffered Locke's solution, the resulting cell volumes being 20% or  $215 \pm 0.1$  million corpuscles per c mm. The experiments are therefore corrected for a standard volume<sup>o</sup>. In all resuspension experiments the syringes were rinsed in 10% potassium oxalate solution.

*Results* Table I records the results, expressed as averages, obtained on 13 different samples of blood.

It will be noted that although the sedimentation rates of the 2 series differ considerably when suspended in their respective plasmas, there are no marked differences when these same cells are resuspended in Locke and acacia solutions under standard conditions of volume.

The following experiment was done in order to determine macroscopically, the time of rouleaux formation, and the rapidity of sedimentation. For this purpose a stop-watch was used.

TABLE II

The Sedimentation Rate Expressed in Minutes for a Selected Sample

S.R. Lenzenmeier technic	235								
S.R. oxalated undiluted specimen	176								
S.R. cells resuspended in plasma E V	50'	RBC's = 2,220,000/cmm.							
S.R. " " " Locke E V	450	RBC's = 2,110,000/cmm.							
S.R. acacia in Locke	Fall in mm	1%	2%	3%	4%	6%	8%	10%	
Rouleaux formation begins at		2 0"	1 10"	1 15"	1 02'	1'03'	2'25"	2' 0"	
	mm								
	1	5 50	1 25		1'15"	1 58'	3 45"		
	2	9 35	2 19	1 30"	2 14"	2'44'	4 15"		
	3	12 20	3 08"			2'55'	4 30	4'50"	
	4	13 50'	4 25			3'10'	4 45"	5'15"	
	5	14 45	4 39"	2'30"	2 39	3'17"	4 57"	5'40"	
	6	17 45"	4 52	2 45"		3'24'	5 10"		
	7	20 30"	5 29"			3 31"	5'19"	6 15"	
	8	23 15"	5 58			3 46'	5'26'		
	9	26 25"	6 27				5'33"		
	10		6 42		3 04		5 40"		
	11		6 50"				5'47"	6 45'	
	12	34 10"	7 20"	3 30	3 19"	4 26	5 54"	6 55"	
	13		7 50"				6 02"		
	14		8 23'						
	15		8 43						7'25"
	16		9 05'						
	17		9 25						
	18	56 0"	10 00"	4 15"	3 54'	4 56"	6 52"	8 10"	

An examination of Table II reveals that at least one minute elapses before rouleaux formation is initiated. Sedimentation begins slowly at first and then progresses with an accelerated velocity followed by retardation. In rapidly sedimenting bloods the rate of sedimentation is not a rectilinear function.

non<sup>4</sup> reveals that although there appears to be an association between an increase in blood serum globulins and a diminished suspension stability the real causative factor is an agglutinant which may or may not be associated with the globulins. The sedimentation of red blood corpuscles in the presence of this agglutinant, found in physiologically altered blood, may be paralleled experimentally in solutions of gum acacia.

When solutions of gum acacia are administered intravenously to human subjects it is noted that the blood becomes difficult to smear, and that the suspension stability of the erythrocytes is diminished. Similar phenomena may be observed when blood is mixed with gum acacia solutions *in vitro*.

**Method** Human subjects with normal and shortened sedimentation rates and subjects receiving gum acacia solutions intravenously were chosen for this study. All sedimentation experiments were done using the Friedlander tube and recording the time necessary for the column of erythrocytes to settle 18 mm. A 20 cc sample of venous blood was withdrawn and oxalated. A sedimentation test was done directly on this sample and another was done using the Lenzenmeier technic. The remainder of the sample was separated by centrifugation and the corpuscular moiety washed with 3 changes of Locke's solution. Then 0.2 cc of washed corpuscles were resuspended in 0.8 cc of the following menstrua plasma.

TABLE I  
Sedimentation Rate Expressed in Minutes for a Column of Cells to Settle 18 mm.

					Slowly Sedimenting Blood 4 samples	Rapidly Sedimenting Blood 9 samples
SR	Lenzenmeier technic				192	24
SR	oxalated undiluted specimen				186	14
SR	cells resuspended in plasma				44	7
SR	"	"	"	Locke	EV	719
SR	"	"	"	1% acacia	EV	60
SR	"	"	"	2% "	EV	11
SR	"	"	"	3% "	EV	47
SR	"	"	"	4% "	EV	5
SR	"	"	"	6% "	EV	65
SR	"	"	"	8% "	EV	92
SR	"	"	"	10% "	EV	36

S.R. = Sedimentation rate

EV = 20% cell volume

<sup>3</sup> Westergren, C., Theorell, H., and Widstrom, G., *Z f d g Exp Med* 1931, 75, 668

<sup>4</sup> Lucia, S. P., Gospe, S., and Brown, J. W., to be published

<sup>5</sup> Lenzenmeier, G., *Arch f Gynaekologie*, 1920, 113, 608

second, normal, mature males, and the third, ovariectomized females castrated some 9 months previously. All animals were injected once per day with 5 rat units of estrin during the first 3 weeks and with 20 rat units for the next week. In the second series, groups of the same size and kind of animals were used. These were given 5 rat units of estrin during the first 3 weeks and 20 rat units daily for the following 5 weeks. The material was highly purified, non-crystalline estrin from human pregnancy urine, carefully assayed by the Coward-Burn method. After the injections were completed the animals were allowed to rest for 4 days to permit excretion of residual estrin. They were then etherized, the carotid cannulated and the blood withdrawn through the cannula into a syringe. The blood was permitted to clot and between 3 and 4 cc of serum obtained per rat.

In determining the ability of this serum to neutralize the effect of estrin, the following procedure was adopted. One hundred newly ovariectomized rats were used, one group was injected with estrin only, one group with estrin plus serum in the same amounts and from rats of the same kind as the injected animals and the third with estrin plus the serum from the injected animals. Those given serum received one-third the total amount at the same time they received the estrin, one-third 12 hours later and the remainder 24 hours after the first injection. The serum given represented approximately the total amount obtained from one donor rat and varied between 3 and 4 cc. The estrin was given in oil subcutaneously and the serum intraperitoneally. All rats were given 2 Coward-Burn rat units of estrin. (One Coward-Burn rat unit repre-

TABLE I

No of Rats Injected		Material Injected					% in Estrus
50	2 R U	Estrin only					88
10	2 "	"	plus	serum	from	non injected males	80
10	2 "	"	"	"	"	males injected for 1 mo	100
10	2 "	"	"	"	"	" " " 2 mo	90
10	2 "	"	"	"	"	non ovariectomized, non injected females	90
10	2 "	"	"	"	"	non ovariectomized females injected for 1 mo	80
10	2 "	"	"	"	"	non ovariectomized females injected for 2 mo	80
10	2 "	"	"	"	"	ovariectomized, non injected females	80
10	2 "	"	"	"	"	ovariectomized females injected for 1 mo	90
10	2 "	"	"	"	"	ovariectomized females injected for 2 mo	

When samples of corpuscles in acacia solutions are examined microscopically, it is noted that rouleau formation is slow and the clumps small in the 1% solution, rapid with large clumps in the 10% solution

*Summary* The factors responsible for alterations in the suspension stability of erythrocytes is independent of the corpuscles. Solutions of gum acacia reduce the suspension stability of the blood. A solution of 3 or 4% of acacia is the minimal amount that will produce the maximal instability of erythrocyte sedimentation. The rapidity of formation and the size of agglutinated erythrocyte masses progresses parallel to the increase in concentration of acacia in solution. The curve of sedimentation for rapidly sedimenting bloods is not a rectilinear function.

7610

### NO Anti-Hormones Against Estrin \*

FRED E D'AMOUR, CHARLOTTE DUMONT AND R G GUSTAVSON

*From the Research Laboratories, University of Denver*

In 1934, Collip indicated that there may exist anti-hormones, or substances produced as a result of continued hormone injection which tend to counteract the effect of the hormone. In the preparations which he used, however, the possibility of protein being present could not, with absolute certainty, be excluded. The so-called anti-hormone might then possibly have been an antibody, although evidence opposing this belief was presented.

It seemed to us desirable to investigate the possibility of anti-hormone production against estrin since we would here be dealing with a substance certainly not a protein, and also because we found in earlier work<sup>1</sup> that continued estrin administration did not cause a continued increase in size of the uterus, but rather the reverse, which might conceivably be explained on the basis of anti-hormone production.

Two series of experiments were performed. In the first, 3 groups of 10 rats each were used, one group normal, mature females, the

\* This investigation was supported in part by a grant from the National Research Council, Committee on Problems of Sex.

<sup>1</sup> Spencer, Jack, D'Amour, Fred E, Gustavson, R. G., *Am J Anat*, 1932, 50,



second, normal, mature males, and the third, ovariectomized females castrated some 9 months previously. All animals were injected once per day with 5 rat units of estrin during the first 3 weeks and with 20 rat units for the next week. In the second series, groups of the same size and kind of animals were used. These were given 5 rat units of estrin during the first 3 weeks and 20 rat units daily for the following 5 weeks. The material was highly purified, non-crystalline estrin from human pregnancy urine, carefully assayed by the Coward-Burn method. After the injections were completed the animals were allowed to rest for 4 days to permit excretion of residual estrin. They were then etherized, the carotid cannulated and the blood withdrawn through the cannula into a syringe. The blood was permitted to clot and between 3 and 4 cc of serum obtained per rat.

In determining the ability of this serum to neutralize the effect of estrin, the following procedure was adopted. One hundred newly ovariectomized rats were used, one group was injected with estrin only, one group with estrin plus serum in the same amounts and from rats of the same kind as the injected animals and the third with estrin plus the serum from the injected animals. Those given serum received one-third the total amount at the same time they received the estrin, one-third 12 hours later and the remainder 24 hours after the first injection. The serum given represented approximately the total amount obtained from one donor rat and varied between 3 and 4 cc. The estrin was given in oil subcutaneously and the serum intraperitoneally. All rats were given 2 Coward-Burn rat units of estrin. (One Coward-Burn rat unit repre-

TABLE I.

No of Rats Injected		Material Injected					% in Estrus
50	2 R.U.	Estrin only					88
10	2 "	" "	plus serum from	non injected males			80
10	2 "	" "	" "	males injected for 1 mo			100
10	2 "	" "	" "	" "	" 2 mo		90
10	2 "	" "	" "	non ovariectomized, non injected females			90
10	2 "	" "	" "	non-ovariectomized females injected for 1 mo			80
10	2 "	" "	" "	non ovariectomized females injected for 2 mo			80
10	2 "	" "	" "	ovariectomized, non injected females			80
10	2 "	" "	" "	ovariectomized females injected for 1 mo			90
10	2 "	" "	" "	ovariectomized females injected for 2 mo			

When samples of corpuscles in acacia solutions are examined microscopically, it is noted that rouleaux formation is slow and the clumps small in the 1% solution, rapid with large clumps in the 10% solution

*Summary* The factors responsible for alterations in the suspension stability of erythrocytes is independent of the corpuscles. Solutions of gum acacia reduce the suspension stability of the blood. A solution of 3 or 4% of acacia is the minimal amount that will produce the maximal instability of erythrocyte sedimentation. The rapidity of formation and the size of agglutinated erythrocyte masses progresses parallel to the increase in concentration of acacia in solution. The curve of sedimentation for rapidly sedimenting bloods is not a rectilinear function.

## 7610

## No Anti-Hormones Against Estrin \*

FRED E. D'AMOUR, CHARLOTTE DUMONT AND R. G. GUSTAVSON

*From the Research Laboratories, University of Denver*

In 1934, Collip indicated that there may exist anti-hormones, or substances produced as a result of continued hormone injection which tend to counteract the effect of the hormone. In the preparations which he used, however, the possibility of protein being present could not, with absolute certainty, be excluded. The so-called anti-hormone might then possibly have been an antibody, although evidence opposing this belief was presented.

It seemed to us desirable to investigate the possibility of anti-hormone production against estrin since we would here be dealing with a substance certainly not a protein, and also because we found in earlier work<sup>1</sup> that continued estrin administration did not cause a continued increase in size of the uterus, but rather the reverse, which might conceivably be explained on the basis of anti-hormone production.

Two series of experiments were performed. In the first, 3 groups of 10 rats each were used, one group normal, mature females, the

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<sup>1</sup> Spencer, Jack, D'Amour, Fred E., Gustavson, R. G., *Am J Anat*, 1932, 50, 129

Poor results were obtained with the following extra lard, liver or egg fat, the unsaponifiable fractions of liver or egg fat, lecithin, and extra wheat germ oil

## 7612 C

## Spontaneous Activity, Direct and Indirect Measures of Sexual Drive in Adult Male Rats

CALVIN P. STONE AND ROGER G. BARKER.

*From the Psychological Laboratory, Stanford University*

Numerous experiments<sup>1-5</sup> have demonstrated a significant relationship between the level of spontaneous activity in male rats, as measured by revolving drums, and the presence or the absence of the testes. Castration is followed by a marked decrease in spontaneous activity. Although the reduction is somewhat less striking when castration is performed on infantile males than when it is performed on fully developed males,<sup>6</sup> there is at either age a decline in spontaneous activity that may be ascribed to the loss of gonadal secretions.

A clear relationship has also been shown to exist between copulatory behavior and the presence or absence of the testes. Young male rats castrated prior to puberty seldom if ever copulate or display aggressive sexual behavior toward receptive females, furthermore, adult males, although copulating for some weeks or even months after castration,<sup>7</sup> soon show a measurable reduction in sexual drive as measured either by direct tests of copulatory frequency or by obstruction tests.<sup>8</sup> These facts indicate that reduction in spontaneous activity and reduction in quantitative expressions of sexual vigor go hand in hand in castrated males when the latter are compared with normal males. They also suggest the possibility of using the revolving drum technique to study sexual drive in normal

<sup>1</sup> Hoskins, R. G., *Am J Physiol*, 1925, **72**, 324.

<sup>2</sup> Wang, G. H., Richter, C. P., and Guttmacher, A. F., *Am J Physiol*, 1925, **73**, 581.

<sup>3</sup> Richter, C. P., and Wislocki, G., *Am J Physiol*, 1928, **80**, 651.

<sup>4</sup> Slonaker, J. R., *Am J Physiol*, 1930, **93**, 307.

<sup>5</sup> Richter, C. P., *Quart Rev Biol*, 1927, **2**, 307.

<sup>6</sup> Richter, C. P., *Endocrinology*, 1933, **17**, 445.

<sup>7</sup> Stone, C. P., *J Comp Psychol*, 1927, **7**, 369.

<sup>8</sup> Nissen, H. W., *Genet Psychol Monog*, 1929, **5**, 451.

sents the amount of estrin necessary to bring 50 out of 100 rats into estrus )

Table I shows the results obtained

*Conclusions* We conclude that the results obtained according to the method used do not indicate the formation of any anti-hormone against estrin

## 7611 P

### Effect of Various Dietary Principles on Lactation in Rats

R. G. DAGGS (Introduced by J. R. Murlin)

*From the Department of Vital Economics, University of Rochester, Rochester, N. Y.*

It has been known for some time that milk production can be materially increased by dietary treatment. This increase was first accomplished by increasing the protein content of the diet, then later by using good quality animal proteins such as liver. The vitamins, especially the B complex, are of considerable importance as well.

In the present study the growth of approximately 350 litters of suckling rats was used as a criterion of milk production. Only the growth period from the fourth to the seventeenth day of life was considered. The logarithmic functions of the daily weight of litters of 6 were plotted against time. The slopes of the resultant curves were compared, and expressed as lactation indices.

The basal diet of the lactating rats consisted of casein, 15 parts, salt mixture, 5, agar agar, 2, starch, 76, lard, 14, wheat germ oil, 5 drops per day, cod liver oil, 6 drops per day, and irradiated yeast 3 gm per day. To this basal ration were added daily supplements of various fractions of liver or egg. When dried substances such as amino acids were used they were incorporated in the basal ration replacing part of the casein.

The lactation promoting factor was found to be present in considerable amounts in the following: liver, egg, water extract of autolysed liver or egg, 25% level of casein, Witte's peptone, blood fibrin, cystine. The best results were obtained when one part of cystine replaced one part of casein in the basal ration. This is a rather low protein diet for a rat, especially a lactating rat, and the results suggest that cystine plays a specific role in stimulating milk production either as such or as a constituent of glutathione which in turn may influence the production of milk.

Poor results were obtained with the following extra lard, liver or egg fat, the unsaponifiable fractions of liver or egg fat, lecithin, and extra wheat germ oil

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The tests\* for copulatory frequency were made a few days after the males were taken out of the revolving drums. Each male was tested between the hours of 8 and 11 p.m. in his home cage with one highly receptive female. The number of copulations and the number of ejaculations were recorded during each sub-test period. The total test consisted of 4 sub-tests, each of 15 minutes duration. The first and second sub-tests came on the same evening with a 20-minute pause between tests, the third and fourth sub-tests were conducted exactly as the first pair of tests and were administered one week later. During the interim between the first and the second pair of tests the males were kept apart from the females.

Tests\* with the Columbia Obstruction Apparatus<sup>9</sup> were begun one week after the last of the direct tests for copulatory frequency. With this technique of measuring sexual drive the number of contacts with the electrified grid and the number of crossings to the incentive compartment are taken as the measures of sexual drive. The tests were conducted on 2 nights one week apart. Each night's test consisted of 2 sub-tests that were separated by a 5-minute pause. The sub-tests were of 10 minutes duration and during this time the male was given complete freedom to cross the grid separating the entrance compartment and the incentive chamber. He was returned to the entrance compartment immediately after his arrival in the incentive chamber without giving him an opportunity to copulate with the female incentive. Prior to the test proper, the males were given ample opportunity to familiarize themselves with all parts of the obstruction apparatus, to learn how to manipulate the one-way door leading to the incentive chamber, and to learn that a receptive female with which they had been given opportunity to copulate prior to the tests was in the incentive chamber.

As a measure of the spontaneous activity of each male we have used the total number of revolutions of the drum made during the 28-day period in which his records were taken. The copulatory vigor is denoted by the total number of intromissions during one hour of testing and by a composite score that was obtained by adding to the number of intromissions 10 points for each ejaculation. The obstruction test records consist of the total number of times the grid was crossed in 40 minutes, and of a combined score consisting of the number of crossings plus the number of contacts with the grid. In Table 1 will be found the raw data of each of the tests. As can be seen, there is a considerable amount of variation in the scores.

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\* These tests are described in more detail in a forthcoming paper Stone, C. P., Tomlin, M. I., and Barker, R. G., *J. Comp. Psychol.* (in press).

males, however, it should be borne in mind that an instrument that adequately registers wide variations in a given phenomenon may be wholly inadequate when fine discriminations are required. The usefulness of revolving drums in studying sexual drive in intact animals must be determined by special experiments.

In the present study, we have determined the relationships between spontaneous activity of 24 male, albino rats confined in revolving drums and their numerical scores on 2 reliable tests of sexual drive, namely, (1) frequency of their copulations in direct tests with highly receptive females, and (2) frequency of their attempts to overcome an obstruction separating them from a receptive female.<sup>9</sup> The males were between 6 and 7 months of age. Their average weight was 280 gm and the range of weights was from 190 to 320 gm. They had been reared on the Steenbock diet, supplemented about twice weekly by lettuce. All were in a very fine state of nutrition and entirely free from skin parasites. When the males were approximately 5 months old they were released in a laboratory room with floor space of approximately 16 square meters. With them were placed 2 dozen adult females in order that they might have unrestricted opportunity for sexual congress. From time to time, non-pregnant females were substituted for the pregnant ones during the period of cohabitation which lasted approximately one month.

Finally, the males were transferred as a group to revolving drums made available through the courtesy of Prof J R Slonaker of the Physiology Department, Stanford University. The males were kept in the revolving drums for a total of 5 weeks, one week devoted to preliminary adjustment to the new cage situation and 4 weeks devoted to the study of spontaneous activity. At the end of each week the males were shifted to different drums in order to distribute at random the unmeasured influences of possible adventitious factors such as small differences in room temperatures and air currents, the proximity of sluggish or active animals that might act as sedatives or as stimulants to certain individuals, or differences in the inertia of the cages themselves. So far as we have been able to ascertain from published reports by Prof Slonaker, the amount of the activity of our males appears to be essentially like that of his males of similar ages that were allowed to keep the same cage for long periods of time.<sup>10</sup>

<sup>9</sup> Jenkins, T N, Warner, L H, and Warden, C J, *J Comp Psychol*, 1926, 6, 361

<sup>10</sup> Slonaker, J R, *Am. J Physiol*, 1926, 77, 503



There is no evidence of a significant relation between the number of revolutions in the drums and scores upon either the obstruction apparatus or the direct test. Applying Fisher's test of the significance of correlation coefficients<sup>11</sup> to the correlation between revolutions and contacts plus crossings, the largest coefficient, it appears that there is a probability of 30 in 100 that such a correlation might arise by chance in an uncorrelated population. Obviously no significance can be attached to a coefficient with such a low reliability.

In contrast to this finding, there is clear evidence of some community of function between the direct copulatory and the obstruction test scores.

The reliability of the scores denoting spontaneous activity has been determined for the present data by correlating the sums of revolutions on the odd with the sums of revolutions on the even days of the total test. This correlation is .80 and becomes .88 when the Spearman-Brown formula is used to obtain an estimate of the reliability of the total test.<sup>12</sup> Previously we have found that the reliabilities of the direct copulatory tests and the obstruction test are, respectively, .91 and .89. In view of the reliability of each of these tests, we may assume that the failure to obtain significant correlations between the revolving drum scores and the other tests cannot be ascribed to errors of measurement of the tests.

In conclusion, then, we find no reliable evidence that there is a true relation between the revolving drum activity of normal adult male rats and the best estimates of sexual aggressiveness now available. As experiments with castrates suggest, however, a relatively low correlation might be found if exceedingly large differences in sexual drive existed among the animals being studied.

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<sup>11</sup> Fisher, R. A., *Statistical Methods for Research Workers*, 3d ed., Edinburgh Oliver and Boyd, 1930, xii+283.

<sup>12</sup> Kelley, T. L., *Statistical Method*, New York Macmillan, 1923, xi+390.

TABLE I

Records of Each Male in the Revolving Drum, upon the Test of Copulatory Frequency, and upon the Obstruction Test

Column 2, total numbers of revolutions for the 28 day period, Column 3, total numbers of intromissions in 4 copulatory sub-tests lasting 1 hour, Column 4, composite score obtained by adding to total number of intromissions 10 points for each ejaculation, Columns 5 and 6, respectively, total numbers of crossings of the electrified grid and sums of crossings and contacts with grid of the obstruction apparatus in 4 sub tests which lasted 40 minutes

Animal No	Drum Revolutions (in 1000's)	No of Copulations	No of copulations+ 10(No ejac.)	No of Crossings	Crossings + Contacts
1	10	74	144	23	61
2	201	82	122	160	192
3	119	62	92	110	166
4	109	80	140	123	173
5	114	89	149	179	193
6	141	34	44	113	174
7	17	77	147	97	138
8	111	62	122	182	202
9	168	46	66	59	118
10	25	0	0	8	9
11	158	60	110	144	203
12	73	53	93	136	203
13	196	64	104	74	94
14	49	103	183	148	156
15	25	18	18	16	20
16	215	66	126	55	145
17	29	45	45	6	20
18	233	52	82	85	133
19	48	53	113	291	300
20	86	50	120	228	252
21	93	30	30	29	38
22	94	57	137	83	100
23	91	50	70	177	187
24	24	55	145	127	152
Mean	101.25	57.50	101.67	110.42	144.17
S D	66.26	21.60	45.72	71.44	73.43

from animal to animal, but there is little tendency for the values from the different tests to vary together. This latter point can be verified by inspecting the correlation coefficients of Table II. In

TABLE II  
Product Moment Correlations.

	Crossings	Contacts + Crossings	Copulations	Copulations + 10(No ejac.)
Revolutions in drum	084	269	177	032
Crossings			402	
Contacts + crossings				539

this table will be found the product-moment correlations between the total numbers of revolutions made in the drums, and scores upon the other tests, here, also, will be found the correlations between the scores made on the copulation and obstruction tests

TABLE II.  
Average Blood Carotene and Cholesterols in Diabetics

Age	Sex	Aver		Upper Limit		Lower Limit		Diet			
		Carotene mg %	Chol mg %	Carotene mg %	Chol mg %	Carotene mg %	Chol mg %	Carb gm	Pro gm	Fat gm	Daily Insulin Units
47	M	318	272	338	308	284	232	250	65	85	30
45	M	216	204	230	223	203	194	200	75	85	30
48	M	337	211	351	229	311	194	250	75	85	75
40	M	207	241	216	250	203	227	180	65	85	15
65	M	337	251	378	294	257	222	180	75	85	none
49	M	277	178	324	225	230	156	220	65	85	8
30	F	212	214	230	227	203	200	200	70	83	50
34	F	131	189	149	217	108	161	280	73	83	15
18	M	220	218	230	263	216	176	200	65	85	60
64	F	135	250	162	296	108	200	220	63	83	8
62	F	090	217	094	253	081	194	150	63	70	none
61	F	205	302	256	378	136	264	200	63	85	"
30	M	284	235	337	312	216	200	160	60	90	28
63	M	346	196	378	221	294	181	165	62	108	none
66	F	302	323	351	347	243	301	150	52	62	"
60	M	176	166	203	222	148	143	200	60	87	22
53	M	144	222	162	248	108	208	142	60	90	none
49	F	482	269	750	329	335	219	174	63	83	12
22	M	284	290	297	306	270	258	245	65	85	40

serum carotene to be 0.109 mg % with a standard deviation of  $\pm 0.104$ . The limits then would be 0.213 mg % and 0.005 mg %. All of our observations fell within these limits so that the figures may be considered significant. In the diabetics the average fasting serum carotene was 0.262 mg % with a standard deviation of  $\pm .112$ . The limits are then 0.374 and 0.150 mg %. Seventy-four percent of the figures fall within the average  $\pm$  the standard deviation. Ninety-five percent fall within twice and 99% within 3 times the standard deviation.

It is clear from these observations that the fasting blood carotene is higher in diabetics than in normals. The average of the diabetics being 0.262 mg % as compared to 0.109 mg % for the normals. The average cholesterol in the normals was 178 mg % as compared to 233 mg % in the diabetics.

\* Dunn, H. L., *Physiol Rev*, 1929, 19, 375

## Fasting Blood Carotene Level in Normal and Diabetic Individuals.

HAROLD BRANDALEONE AND ELAINE P. RALLI

From the Department of Medicine, University and Bellevue Hospital Medical College and the Third (New York University) Medical Division, Bellevue Hospital

In the course of a study on the effect of the oral administration of carotene on the blood carotene of normal and diabetic individuals, we investigated the fasting blood carotene level in these 2 groups. The number of figures available on this subject are still somewhat limited, and are mostly single observations taken at random.<sup>1,2,3</sup> Rabinowitch<sup>4</sup> in a study of carotinemia in diabetes does not report the figures for blood carotene as mg % but in units, so that the actual figures are difficult to compare with those of other observers.

The diabetic patients studied were on a known diet of carbohydrate, protein and fat. The normals were on a mixed diet from which carrots were omitted, as was the case in the diabetic diets. Fasting bloods were taken. The serum carotene was estimated by the method of White and Gordon<sup>2</sup> and is reported in mg %. Blood cholesterols were done by the Bloor colorimetric method.<sup>5</sup> Nine normals were observed (Table I) and 19 diabetics (Table II). At least 3 determinations were done on each patient. In all, 35 observations were made on normals and 83 on diabetics. The results on the normals analyzed according to Dunn<sup>6</sup> show the average fasting normal

TABLE I  
Average Blood Carotene and Cholesterol in Normals.

Age	Sex	Average		Upper Limit		Lower Limit	
		Carotene mg %	Chol. mg %	Carotene mg %	Chol mg %	Carotene mg %	Chol mg %
50	M	122	158	122	172	122	148
45	M	077	176	081	192	068	164
48	M	122	190	122	217	122	176
44	M	108	163	108	227	108	145
22	M	136	172	122	179	095	167
34	M	054	105	054	180	054	152
22	M	113	176	135	208	108	150
26	M	148	172	162	192	122	161
22	M	153	161	176	217	122	122

<sup>1</sup> Connor, C. L., *J. Biol. Chem.*, 1928, **77**, 619

<sup>2</sup> White, F. D., and Gordon, E. M., *J. Lab. and Clin. Med.*, 1931, **32**, 17, 53

<sup>3</sup> Stoner, W. C., *Am. J. Med. Sc.*, 1928, **175**, 32

<sup>4</sup> Rabinowitch, I. M., *Arch. Int. Med.*, 1930, **45**, 586

<sup>5</sup> Bloor, W. R., Pelkan, J. F., and Allen, D. M., *J. Biol. Chem.*, 1922, **52**, 191

water) They were weighed before immersion and after intervals of 3, 6, 10, 15, 25, 40, and 60 minutes on a torsion balance, which allows very rapid weighing There is little point in continuing observations beyond about 60 minutes, for the swelling curves become very irregular

The average results for 40 muscles from 20 adrenalectomised animals and for 40 muscles from 20 controls are shown in Table I

TABLE I.

	% gain in weight						
	3	6	10'	15'	25	40'	60'
Normals	106.1	109.1	111.8	114.7	118.3	122.8	127.1
Adrenalectomised	104.1	106.9	109.1	112.1	114.9	118.4	122.3
Difference	2.0	2.2	2.7	2.6	3.4	4.4	4.8
S.E. of difference	0.57	0.68	0.87	1.10	1.28	1.37	1.43

The table shows that the muscles of the adrenalectomised rats take in water at a slower rate than do those of the control animals, a conclusion which is exactly the opposite from that of Winter and Hartman Winter and Hartman do not give figures to show that the differences in swelling rate which they observed (in the opposite direction) were statistically significant, but there is no doubt about the differences shown above If differences in the form of the swelling curves obtained in experiments such as these are to be taken as indicating differences in permeability, our conclusion would have to be that the muscles of adrenalectomised animals are less permeable to water than those of normal animals. there are, however, several reasons for attributing the differences to other than permeability changes

1 Throughout the experiments we observed that heavier muscles tended to swell less rapidly than lighter ones, even when the swelling was expressed in each case as a percentage of the initial weight. This is to be expected on the grounds that the heavier muscles have a smaller surface/volume ratio It so happened that our control muscles were somewhat lighter on the average than the muscles from the adrenalectomised animals, and this difference in weight is probably partly responsible for the slower swelling of the latter

2 When muscles of equal weight from normal and adrenalectomised animals were compared, the rate of swelling of the control muscles was again found to be greater than that for the muscles from the adrenalectomised rats, although the differences were smaller than before Taken individually, indeed, (as in the table

## Swelling of the Muscles of Adrenalectomised Rats \*

ERIC PONDER AND ROBERT GAUNT

*From the Biological Laboratory, Cold Spring Harbor, and The Department of Biology, College of Charleston, S C*

Following a suggestion of Viale and Bruno,<sup>1</sup> that the changes in water content of the tissues of adrenalectomised animals may be due to an increase in permeability, Winter and Hartman<sup>2</sup> carried out experiments on the swelling and shrinking of muscles from normal and adrenalectomised rats in balanced salt solutions of various strengths. They conclude that water enters or leaves muscles from adrenalectomised animals more readily than it leaves or enters normal muscles, and regard this as evidence of an increased permeability following the adrenalectomy. Since a hormonal control of tissue permeability, if demonstrated, would be of extreme importance in general physiology, we have attempted to reproduce Winter and Hartman's results.

The method used was essentially that of Winter and Hartman, except that we confined ourselves to determining the course of the swelling curves of muscles in a hypotonic solution, instead of investigating shrinking curves in hypertonic solutions as well. The latter are notoriously irregular, and are not suitable for analysis. The rats used were adrenalectomised at 30 days of age, and in all but a few cases were killed in the terminal stages of adrenal insufficiency, as indicated by a fall in weight and body temperature, asthenia and oftentimes prostration. In a few cases the rats were killed in slightly earlier stages of adrenal insufficiency. The animals survived from 3 to 32 days after the operation, the average time of survival being 9 days. The average weight at the time they were killed was 63 gm. The control rats were from the same stock, and as nearly as possible of the same age.

The animals were killed by a blow on the head, and both gastrocnemii dissected out entire. The muscles were immersed in the Locke's solution described by Winter and Hartman (pH = 7.3), diluted so as to be hypotonic (70 parts solution plus 30 parts of

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\* We are indebted to Mr. C. E. Tobin for assistance in the preparation of animals.

<sup>1</sup> Viale, G., and Bruno, A., *Compt. Rend. Soc. de Biol.*, 1927, **97**, 261.

<sup>2</sup> Winter, C. A., and Hartman, F. A., *Proc. Soc. Exp. Biol. and Med.*, 1933, **31**, 207.

water) They were weighed before immersion and after intervals of 3, 6, 10, 15, 25, 40, and 60 minutes on a torsion balance, which allows very rapid weighing There is little point in continuing observations beyond about 60 minutes, for the swelling curves become very irregular

The average results for 40 muscles from 20 adrenalectomised animals and for 40 muscles from 20 controls are shown in Table I

TABLE I.

	% gain in weight						
	3'	6'	10'	15'	25	40'	60'
Normals	106.1	109.1	111.8	114.7	118.3	122.8	127.1
Adrenalectomised	104.1	106.9	109.1	112.1	114.9	118.4	122.3
Difference	2.0	2.2	2.7	2.6	3.4	4.4	4.8
S.E. of difference	0.57	0.68	0.87	1.10	1.28	1.37	1.43

The table shows that the muscles of the adrenalectomised rats take in water at a slower rate than do those of the control animals, a conclusion which is exactly the opposite from that of Winter and Hartman Winter and Hartman do not give figures to show that the differences in swelling rate which they observed (in the opposite direction) were statistically significant, but there is no doubt about the differences shown above If differences in the form of the swelling curves obtained in experiments such as these are to be taken as indicating differences in permeability, our conclusion would have to be that the muscles of adrenalectomised animals are less permeable to water than those of normal animals there are, however, several reasons for attributing the differences to other than permeability changes

1 Throughout the experiments we observed that heavier muscles tended to swell less rapidly than lighter ones, even when the swelling was expressed in each case as a percentage of the initial weight. This is to be expected on the grounds that the heavier muscles have a smaller surface/volume ratio It so happened that our control muscles were somewhat lighter on the average than the muscles from the adrenalectomised animals, and this difference in weight is probably partly responsible for the slower swelling of the latter

2 When muscles of equal weight from normal and adrenalectomised animals were compared, the rate of swelling of the control muscles was again found to be greater than that for the muscles from the adrenalectomised rats, although the differences were smaller than before Taken individually, indeed, (as in the table

above) only one such difference exceeded twice its standard error, but all the differences were in the same direction, and the odds against this occurring by pure chance are enormous. A simple explanation exists, however, for this greater rate of swelling in the case of the controls, for the muscles from the control animals contained a smaller percentage of water than those of the operated animals<sup>3-5</sup>. If we suppose that the initial tonicity of the control muscles was higher than that of the muscles of the adrenalectomized animals, the former would be expected to swell more rapidly than the latter in any given hypotonic solution. The average water content of our control muscles was 76.6%, and that of our muscles from the operated animals 78.3%, this difference is about of the right order, if reflected in a tonicity change, to account for the different swelling rates observed.

3 While these are the average results for all the muscles used, the swelling curves of individual muscles vary so much that we are very doubtful if this type of experiment can be relied upon to supply information regarding permeability of tissues. For instance, the muscles of the right and left legs of the same animal do not always show the same swelling curve, even when the 2 muscles are of the same initial weight, and we have met with as great differences between the behavior of the paired muscles as between the behavior of normal muscles and those from the adrenalectomized animals.

Finally, it ought to be pointed out that an increase in the permeability of the tissues to water, and this alone, could not account for the increased water content of the tissues after adrenalectomy. The water content is determined, not by the rate of entry of water, but by those conditions which control the final equilibrium, and these might well change without any alteration in permeability being involved.

*Conclusions* The conclusion of Winter and Hartman, that adrenalectomy in the rat is followed by an increase in the rate at which water enters the muscles, i. e., by an increase in permeability, is not confirmed. The observation that the muscles of the operated animals contain a greater percentage of water is confirmed. It is pointed out that an increase in permeability to water, even if it were to exist, would not account for the increased water content.

<sup>3</sup> Hartman, F. A., Brownell, K. A., and Lockwood, J. E., *Endocrinology*, 1932, 16, 521.

<sup>4</sup> Silvette, H., and Britton, S. W., *Am. J. Physiol.*, 1933, 104, 399.

<sup>5</sup> Hartman, F. A., *Annals Int. Med.*, 1933, 7, 6.



# Effect of Feeding Thyroid on Anterior Hypophysis of the Female Albino Rat \*

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It has been reported that feeding thyroid to rats increases the gonad-stimulating complex of the anterior hypophysis<sup>1 2</sup> In an attempt to obtain a morphologic basis for this increased physiologic effect, we have studied serial sections of the anterior pituitaries of 28 female rats which were fed varying amounts (250 to 1,000 mg ) of thyroid daily for periods of 30 to 71 days Twelve of these rats were placed on experiment when immature, the others were fully mature when thyroid feeding was initiated Confirming studies of other investigators,<sup>2 3 4</sup> it was found that feeding thyroid in sufficient amounts suppressed the oestral cycle resulting in prolonged periods of dioestrus However, it was also found that by varying the dosage of thyroid the oestral cycle could be regulated almost at will In rats receiving the smaller amounts, the oestral cycles were usually regular, occasionally interspersed with cycles the length of pseudo-pregnant cycles Weichert and Boyd<sup>5</sup> consider that such cycles represent a true lutein phase Larger dosage resulted in prolonged periods of dioestrus, ranging up to 40 days In some animals, oestrus was suppressed for periods of time ranging up to 72 days, which covered the entire period of observation. The heavier thyroid dosages fed in these experiments were sufficient to decrease the growth rate slightly, but never to a marked degree Animals receiving smaller dosages exhibited a normal rate of growth

At autopsy it was found that the weight of the ovaries and the pituitaries of those animals which had exhibited oestral cycles of normal or pseudo-pregnant length were normal in appearance and weight In those rats in which the oestral cycle had been suppressed to a more marked degree, the ovaries were decreased in weight and

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\* These studies were aided by grants from the Committee for Scientific Research of the American Medical Association and the Division of Medical Sciences of the Rockefeller Foundation.

<sup>1</sup> Evans, H. M., and Simpson, M. E., *Anat Rec*, 1930, **45**, 215 (Supplement)

<sup>2</sup> Van Horn, H. N., *Endocrinol.*, 1933, **17**, 152

<sup>3</sup> Reiss, M., and Pereny, S., *Endokrinologie*, 1928, **2**, 181.

<sup>4</sup> Weichert, C. K., *Physiol Zool*, 1930, **3**, 461

<sup>5</sup> Weichert, C. K., and Boyd, R. W., *Anat Rec*, 1933, **58**, 55

the accessory reproductive organs approached a castrate condition. In these rats the weight of the pituitary was also decreased. Considering the group as a whole, the mean pituitary weight was 7.8, while that in 69 control females was 10.9 mg.

The pituitaries were prepared for study by means already described.<sup>6</sup> The ovaries and accessory organs were fixed in Bouin's fluid and the sections stained with hematoxylin and eosin. Serial sections of the pituitary and the ovaries were cut. Cell counts were made on representative sections of the anterior pituitary, a total of 89,535 cells was counted.

Histologically the ovaries and the accessory organs of those animals in which the oestral cycles were moderately to markedly suppressed exhibited definite changes. The ovaries exhibited a normal number of follicles, mostly small and moderate in size, but a few were as large as those found in the ovaries of late prooestral rats. However, much follicular atresia was evident which involved most of the follicles. The large amount of interstitial tissue and the small number of corpora lutea were the outstanding characteristics. Some rats exhibited only one or 2 periods of oestrus, or none at all during the 71 days of observation. In the ovaries of these animals corpora lutea were almost entirely absent, only follicles and interstitial tissue being present. The uteri and vaginae of these rats approached a castrate condition.

Histologically the anterior pituitaries presented certain changes which varied from normal to a degree proportional to that in which the oestral cycle and the reproductive tract varied from normal. The basophiles were increased very slightly in percentage, the mean level in the experimental rats was 5.3%, while the mean level in 69 virgin control rats was 4.8%.

The basophiles were consistently different in appearance from those found in normal females. They were larger and well filled with granules, which took a purple-red stain, varying to a brick red. The granules of basophiles in the anterior pituitaries of normal females take a deep blue stain and often are so closely packed that they give the impression of a dense blue cytoplasm. It is important to emphasize that the basophiles in the anterior pituitaries of the thyroid-fed rats were packed full of granules, while in normal rats these cells exhibit varying degrees of granular depletion. The changes in the basophiles described above were remarkably constant and were most marked in rats in which the oestral cycle had been most markedly suppressed.

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<sup>6</sup> Cleveland, R., and Wolfe, J. M., *Anat. Rec.*, 1932, 51, 409.

In 8 rats which exhibited normal oestral cycles of 5 or 6 days in length, or cycles of pseudopregnant length, the level of the eosinophiles ranged from 30 to 40%, which is the normal level (the mean level of these cells in 69 controls was 33.6%, with a standard deviation of 4.5). The remaining 20 experimental rats exhibited varying degrees of suppression of the oestral cycle. In the anterior pituitaries of these rats the level of the eosinophiles ranged from 24.1 to 28%, such a low level is found only occasionally in normal females.

It is known from the work of the investigators mentioned above that the capacity of the anterior hypophysis to increase the size of the ovaries of immature test animals is increased by thyroid feeding. Our morphologic studies would indicate that there were definite structural changes in the anterior pituitaries of such rats, most notable was some increase in size and definite increase in granular content and change in appearance of the basophilic elements. Changes in the eosinophiles were less constant and where such changes did occur, they were in the nature of a decrease in the percentage of these cells. Our data would indicate, therefore, that the increased capacity of the anterior pituitaries of thyroid-fed rats to increase the weight of the ovaries of immature test rats was probably due to the changes in the basophiles noted above.

One interesting point should be noted, the basophiles in these animals are very similar to those found in the anterior pituitaries of rats killed during the last half of pregnancy. As pointed out previously,<sup>7</sup> during the first 6 days of pregnancy the basophiles are markedly reduced in percentage and granular content, but from the 7th day to delivery they increase gradually in percentage and size and become filled with purple-red granules which vary to brick-red in color. The reason for the similarity of the appearance of the basophiles in these 2 groups of rats is unknown.

The mechanism by which thyroid feeding disturbs the oestral cycle has been discussed fully by Van Horn and Weichert and Boyd. The view that the increased metabolic rate in some way keeps the level of oestrin below the threshold for oestrus is upheld by a certain amount of evidence presented by these workers and by Reiss and Pereny.

*Summary* Twenty-eight female rats were fed amounts of desiccated thyroid ranging from 250 to 1,000 mg daily. The oestral cycle was suppressed to varying degrees, dependent on the dosage of thyroid. The pituitaries were subnormal in weight. Histologically the anterior lobes exhibited certain changes most notable was

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<sup>7</sup> Wolfe, J. M., and Cleveland, R., *Anat. Rec.*, 1933, 50, 33

a slight increase in the percentage of the basophiles and a definite increase in the size and granular content of these cells. The granules stained a purple-red which varied to a dull brick-red, in normal female rats (virgin and killed during the normal oestral cycle) the basophiles take a deep blue stain. The changes in the basophiles were most marked in those animals in which the suppression of the oestral cycle was most evident.

## 7616 C

## Variations in Contour of the Records Found in Serial Electrocardiograms of the Dog

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In the course of some studies we had occasion to take serial electrocardiograms on 3 normal dogs twice a week over a period of 4 months. The dogs were trained to lie on their right sides while the electrocardiograms were taken. The limbs were shaved and flannel bandages soaked in concentrated saline bound around them. Copper wire spiral was then applied tightly over the bandage and connected with the electrodes. The skin resistance was found to be low with this procedure (below 1000 ohms) and no polarization was encountered.

The serial records obtained in each dog over the period of 16 weeks revealed irregular fluctuations in the form of the electrocardiograms. These variations were not progressive, could not be related to environmental factors, and varied within the wide limits illustrated in Fig. 1. These results were obtained in spite of the fact that every effort was made to take the successive electrocardiograms under identical conditions as regards the position and posture of the animal. These normal variations in the electrocardiogram of the dog are probably due to variations in the position of the heart at different times. The relative mobility of the dog's heart as compared to the human is such that it is almost impossible to manoeuvre it into exactly the same position time after time. These effects of position involve changes in the amplitude and even direction of all the complexes of the electrocardiogram, especially the T wave. Sim-

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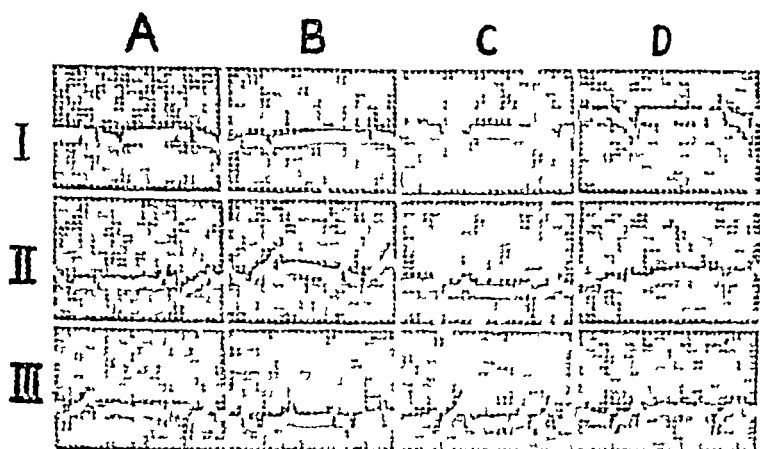


FIG 1

Electrocardiograms showing the range of variation observed in a normal dog, under identical conditions, during a period of 16 weeks. These records (segments A-D) are typical of a large number.

ilar variations in the electrocardiogram of one of these animals were observed when repeated records were made with the animal in the normal standing position.

*Conclusion* Repeated electrocardiograms in the normal unanesthetized dog involving repeated preparation of the animal for this procedure, show significant variations. These variations are probably accounted for by the relative mobility of the dog's heart as compared to the human, and must be taken into account in the interpretation of the results of experiments requiring repeated records over a period of days.

# Behaviour of Isotonic and Hypertonic Solutions in Blood Stream of Normal and Dehydrated Animals

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The mode of regulation of the blood volume remains still an unsolved problem in spite of the efforts of many investigators<sup>1-4</sup> It has been pointed out recently that the blood stream does act to some extent as a reservoir in the normal animal This study was undertaken to evaluate the role played by this reservoir action in the regulation of the blood at a constant volume and the effects of dehydration and protein loss The investigation was divided into 2 parts, the first dealing with effects produced by isotonic solutions introduced in large amounts into the blood stream of normal and dehydrated animals The second part concerns the results produced by hypertonic solutions

## *The effects of isotonic solutions*

*Material used*—10 normal healthy dogs Five dogs were used as controls and were allowed free access to water and food Five were dehydrated by deprivation of water for a standard period of 10 days but food was permitted freely

*Method* 970-980 cc of 0.9% sodium chloride solution or of 5% glucose solution were injected into the blood stream at a constant rate of 330 cc per 5 min Five cc of blood were withdrawn from the femoral vein before and immediately after injection and at intervals of 15, 60, 180 and 300 minutes The hemoglobin was determined by the Newcomer method, the plasma volume by the hematocrit method and erythrocyte counts were performed

*Results* The blood vascular system does act as a reservoir, the blood remaining diluted for a period of 5 hours, at which time it has almost returned to its normal state With isotonic glucose solutions,

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\* Jessie Horton Koessler Fellow in Experimental Surgery

<sup>1</sup> Bogert, L J, Underhill, F P, and Mendel, L B, *Am J Physiol*, 1916, **41**, 189

<sup>2</sup> Smith, A H, and Mendel, L B, *Am J Physiol*, 1920, **53**, 323

<sup>3</sup> Chanutin, A., Smith, A H., and Mendel, L B, *Am J Physiol*, 1924, **68**, 444

<sup>4</sup> Erlanger, J, *Physiol Rev*, 1921, **1**, 177

<sup>5</sup> Calvin, D B, Smith, A. H., and Mendel, L B, *Am J Physiol*, 1933, **105**

the blood dilution shows a progressive diminution to the normal, *i. e.*, there is a uniphasic response. With isotonic sodium chloride solutions, the response is polyphasic, *e. g.*, the maximum dilution may not be reached until one hour after the injection or the initial dilution is followed by concentration and later by a further dilution. The blood of the normal animal retains sodium chloride solutions better than glucose solutions. Dehydration definitely increases the ability of the blood to hold both isotonic sodium chloride and isotonic glucose. Animals, which, by repeated bleedings, have been rendered anemic, as indicated by a definite lowering of the amount of hemoglobin in the blood, are unable to hold water in the blood vascular system even when severely dehydrated. In the normal animal the changes in blood dilution follow closely the changes in urinary secretion. In dehydration, however, following an injection, the blood dilution continues to diminish even although no urine is being excreted, indicating the passage of fluid into the tissues. This study seems to indicate that the major site of electrolyte action is in the extravascular tissues rather than in the blood, and that the blood colloids play a greater role than do electrolytes in the maintenance of a constant blood volume whereas, in the tissues, water storage seems to depend to a greater extent upon electrolyte action.

#### *Effects of hypertonic solutions*

*Material*—20 dogs. 10 dogs were used as controls and 10 were dehydrated.

*Method*. Both femoral veins or external jugular veins were exposed under local anesthesia. Morphine hydrochloride was administered before each experiment. The standard injection was 50 cc of 25% sodium chloride solution. Five cc of blood were withdrawn from opposite vein before, and at intervals of  $\frac{1}{2}$  minute, 3, 7, 10, 15, and 30 minutes following injection. Hemoglobin determination by Newcomer method. Red blood cell count and plasma volume by hematocrit method.

*Results*. The effect of the injection is instantaneous and results in an increase in the volume of circulating blood. This increase varies from 15.2% to 38.9% according to hemoglobin dilution measurements. The volume increase is of short duration and the norm is reached within 20-30 minutes. In the dehydrated animal the blood volume increase is just as great as in normal animals. Often the response is even greater in the dehydrated state. In dehydration the return of the diluted blood to normal is definitely slower. Repeated hemorrhages leading to a loss of the cellular and protein content of the blood results in a considerable diminution in the response to the standard injection.

# Microscopic Observations on Circulatory Systems of Living Transilluminated Mammalian Spleens and Parturient Uteri \*

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*From Hull Anatomical Laboratory, The University of Chicago*

A Spleens—The circulatory system of living spleens of mice, rats, and cats, were studied. The spleens were exposed through a small incision and *vigilantly* protected from minute temperature changes and trauma to prevent general vasomotor upsets in the organ. The spleens were transilluminated, using only the visible spectrum, by means of the previously described<sup>1</sup> quartz rod light. The linings of blood vessels, including sinuses, in living spleens, show as clear retractile borders, not to be confused with peripheral plasma layers of the blood. These linings are as continuous in living spleens as in other organs. Each vessel that I traced connected to both the arterial and the venous system, neither open ends nor blind ends of vessels were found. I saw blood pass through the red pulp via (1) long straight capillaries, (2) via the venous sinus systems and (3) via diapedesis, but I have not yet seen other types of passage. The distribution of blood to various areas and sub-areas of the red pulp is actively controlled by coordination of the action of powerful sphincter-like segments of branches of the arterial tree. Venous sinuses have a cycle of filling, storage and emptying. During filling the efferent end of the sinus is tightly contracted, whole blood flows into the sinus, plasma filters rapidly out of the sinus into the partitions which are usually termed pulp cords, leaving the sinus distended up to 20 to 50 times its original diameter with solidly packed blood cells. The retention of blood cells lasts from a few minutes to several hours. At emptying, the efferent end of the sinus relaxes suddenly, the packed blood cells emerge in masses, and the sinus decreases in diameter, quickly, until it is but 2 or 3 times the diameter of a red blood cell and then it conducts blood like any other blood vessel. The spleens of digesting animals are large because many sinuses are distended with packed cells. The spleens of exercised or frightened animals are small because most of the sinuses are not storing, but conducting, blood. Administra-

\* This research was aided by a grant to The University of Chicago by the Rockefeller Foundation. The assistance and counsel of the members of the Hull Anatomical Laboratory have been invaluable in this work.

<sup>1</sup> Kniseley, M. H., *Anat. Rec.*, 1934, 58, 73



tion of adrenalin causes the sinuses to empty out their stored blood cells

During the brief death period of an animal there is a rapid diapedesis of red cells, out of capillaries, in all directions through the red pulp, a disappearance of capillary walls, an intense phagocytosis of red cells, by phagocytes, and rouleaux formation in the sinuses and venules. Agonal changes in the red pulp during the 3 to 5 minutes of the death of the animal may possibly explain the "open" circulatory system as seen in some histologic sections

B Parturient uteri.—The living uteri of 6 parturient house mice (*Mus musculus*) were studied, using lens combinations with magnifications up to 100 X. The animals were opened, under light ether, without blood loss, by a para-midline incision, the viscera protected *carefully* from thermal and mechanical trauma, and the uteri and adnexa transilluminated with the quartz rod light. Each of the 2 to 5 branches of the uterine artery supplying a placental site has an especially contractile segment (similar to that in an arteriovenous anastomosis) located near the entrance of the branch into the uterine wall. Contraction and relaxation cycles of these sphincters control the volume of blood supplied to a placental site preceding, during and following parturition. At the site of the lowest attached foetus brief partial contractions of the arterial sphincters alternate with long relaxations. Gradually the duration and degree of contraction change until contractions and relaxations are equal and each contraction completely obliterates its branch's lumen.

The sphincter's contractions later become long and powerful with very brief partial relaxations. The foetus and foetal side of the placenta at this time are cyanotic while the maternal side of the placenta and uterine wall are blanched and nearly bloodless. The uterine musculature at the level of the attached placenta (Rudolph & Ivy)<sup>2</sup> begins powerful rhythmical contractions. (Smooth muscle of the gut also undergoes strong contractions when its blood supply is cut off.) During a strong, *localized* uterine contraction the foetus and placenta break away and start down the tube, and the sphincters remain tightly closed, preventing hemorrhage. Brief partial relaxations prevent blood loss while a clot is formed at the placental site. The relaxations of the sphincters become longer and the contractions shorter until after half an hour when a clot is well established the uterus receives a normal blood supply again.

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<sup>2</sup> Rudolph and Ivy, *Am J Obstet and Gynec*, 1930, 19, 317

The cycles of adjacent sphincters are out of phase at all times so that the uterine musculature frequently gets a *little* blood through each, even during the detachment of the foetus and placenta. In so small an animal the loss of a little blood is serious. This mechanism conserves blood so well that free blood is hardly ever seen in the uterine lumen. The *localized* interruption of the blood supply of the uterus at a placental site may be one link in the chain of events initiating delivery.

## 7619 C

## Anterior Pituitaries of Infantile Female Rats Receiving Injections of Pregnancy Urine Extract \*

J M WOLFE

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Many investigators have demonstrated that injections of pregnancy urine or human placental extracts into immature (21-day or above) female rats result in an increase in the size of the ovaries due to follicular maturation and corpus luteum formation. However, subsequent studies of Selye and Collip<sup>1</sup> have revealed that injection of such extracts into infantile female rats (6 to 8 days) fails to cause follicular maturation and development of corpora lutea, but does result in a marked increase in the size of the thecal cells giving rise to thecal corpora lutea.

Collip and associates<sup>2</sup> have found that injection of placental extracts increases the size of the pituitaries of immature female rats (21 days or above) as well as the ovaries. We have confirmed these results using both extracts of human placentae and pregnancy urine.<sup>3-5</sup> Histologically, the pituitaries of these rats exhibited a

\* These studies were aided by grants from the Committee for Scientific Research of the American Medical Association and from the Division of Medical Sciences of the Rockefeller Foundation.

<sup>1</sup> Selye, H., and Collip, J. B., *Proc Soc Exp Biol. and Med.* 1933, **30**, 647.

<sup>2</sup> Collip, J. B., Selye, H., Thomson, D. L., and Williamson, J. E., *Proc Soc Exp Biol. and Med.*, 1933, **30**, 590.

<sup>3</sup> Pregnancy urine extract, Follutein, was furnished by E. R. Squibb & Sons through the courtesy of Dr. J. J. Durrett.

<sup>4</sup> Wolfe, J. M., Phelps, D., and Cleveland, R., *Proc Soc Exp Biol. and Med.*, 1933, **30**, 1092.

<sup>5</sup> Wolfe, J. M., *Proc Soc Exp Biol. and Med.*, 1934, **31**, 812.

<sup>6</sup> Wolfe, J. M., *Am J Physiol.*, in press.

marked granular loss from the basophiles and a less evident loss of granules from the eosinophiles. Cell counts revealed that the percentages of the basophiles and eosinophiles were decreased, while that of the chromophobes was increased. Since it has been found that injection of pregnancy urine extract brings about a markedly different ovarian effect in infantile rats (6 to 8 days) it seemed of interest to study the anterior pituitaries of such rats.

Litters of female rats 6 days old were used, 2 or 3 animals of each litter serving as experimental animals, the rest as controls. The experimental rats received 25 units of pregnancy urine extract daily. Twenty-four hours after the 10th daily injection one experimental and one control animal were autopsied. The remaining experimental rats received 10 more daily injections and, together with their controls, were sacrificed on the 27th day of life, 24 hours after the 20th injection. Due to the small size of some of the litters, all the controls of this group were not littermates. A total of 64 rats was used. At autopsy, body, ovary and pituitary weights were obtained. The ovaries and accessory reproductive organs were fixed in Bouin's fluid and prepared for study, while the pituitaries were fixed in Regaud's fluid and stained by methods previously described.

The various weights are recorded in Table I. After 10 daily injections the ovaries of the experimental rats were increased to a mean weight of 13.4 mg, while that of the controls was only 6.1 mg. After 20 daily injections the ovaries of the experimental animals were increased to a mean weight of 62.4 mg, while the mean weight of the ovaries of the controls was 17.2 mg. These injections failed to increase the weight of the pituitaries of the experimental rats over those of the controls (Table I). This is in direct contrast to the findings when such injections are carried out in 21-day rats. Histologic examination of the ovaries of the experimental rats revealed that follicular maturation and corpus luteum formation had not occurred, but there was a marked hypertrophy of the thecal cells resulting in thecal luteinization. This process, as usually well under way, in the rats killed after 10 injections and as marked in the rats killed after 20 days.

Serial sections of all pituitaries were prepared for study and cell counts made. Comparison of the anterior pituitaries of the experimental rats, killed after 10 injections, with those of their controls, revealed that the level of the eosinophiles was practically the same in the 2 groups (Table I). In the controls the level of the basophiles was high and a great majority of the cells were filled with

TABLE I

The quantitative data pertaining to the percentages of the various cell types are given in a frequency distribution table. Class intervals are given in percentage. Mean rat, ovary and pituitary weights, as well as number of rats used, are given below.

Class Intervals %	25 U Follutein daily—10 days		25 U Follutein daily—20 days	
	Control	Exp	Control	Exp
Eosinophiles				
30-34.9	1	3	9	
35-39.9	11	8	7	
40-44.9	3	2	5	8
45-49.9		3		4
Basophiles—Granular				
0-1.9		16		12
2-3.9			1	
4-5.9	2		2	
6-7.9	6		8	
8-9.9	7		10	
Basophiles—Non granular				
0-1.9	12	5	9	5
2-3.9	3	6	12	7
4-5.9		5		
Chromophobes				
45-49.9		1	3	
50-54.9	6	4	7	
55-59.9	9	3	11	4
60-64.9		8		8
Total Rats per Group	15	16	21	12
Mean Rat Wt (gm.)	29.1	28.6	50.2	54.2
Mean Ovary Wt (mg.)	6.1	13.4	17.2	62.4
Mean Pituitary Wt. (mg.)	1.96	1.96	2.4	2.6

granules, while in the experimental rats the percentage of the basophiles was markedly decreased and those present were regressive and contained few if any granules. The chromophobes were slightly more abundant in the experimental rats. The quantitative data are given in Table I.

Comparison of the anterior pituitaries of the experimental rats, killed after 20 injections, with those of their controls shows that in the experimental animals the level of the basophiles was still very low and those present were practically devoid of granules. On the other hand, the level of these cells in the controls was much higher and a majority were well filled with granules. However, of greater interest was the high level of the eosinophiles in the injected animals of this group, which in every instance was above 40%. This was a moderate but a well defined increase over the level usually found in the controls (Table I). The eosinophiles in the anterior pituitaries of the controls and experimentals were morphologically similar, although in some of the experimental rats they were slightly larger and the negative image of the Golgi apparatus was more prominent.

## 7620 C

## Diurnal Variation in Blood Sugar Level of the Rat.

M. CAROLINE HRUBETZ (Introduced by H. B. Williams)

*From the Department of Physiology, College of Physicians and Surgeons, Columbia University*

It has been the custom in this laboratory to begin all blood sugar experiments on the rat at 9 00 A. M. This procedure has been adopted because it has never been shown whether or not the blood sugar level of the rat varies or remains constant throughout the day. Scott<sup>1</sup> has shown that "when other conditions are maintained as constant as is practicable, the blood sugar in the rabbit is independent of the time of day, at least during the ordinary working hours." It is the purpose of this paper to show the relative blood sugar levels of rats throughout the day.

A total of 200 observations were made on 192 normal-fed rats. The animals were fed *ad libitum* to the time of removal from their cages for bleeding. Approximately 50 observations were made at each of the following hours: 9 00 A. M., 12 00 noon, 3 00 P. M., and 6 00 P. M. The Somogyi micro-method,<sup>2</sup> a modification of the Shaffer-Hartmann method,<sup>3</sup> was used.

The entire experiment was completed in 7 days thus reducing the probability of interference of environmental changes or of changes in the conditions of the animals. This is reflected in the constancy of the results as shown by the very small deviations. These results justify the conclusion that the blood sugar level of the rat is independent of the time of day, at least during the ordinary working hours.

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<sup>1</sup> Scott, E. L., *Arch. Int. Med.*, 1929, **43**, 393.

<sup>2</sup> Somogyi, M., *J. Biol. Chem.*, 1926, **70**, 599.

<sup>3</sup> Shaffer and Hartmann, *J. Biol. Chem.*, 1920, **43**, 349.

## Epinephrine and the Blood Sugar Level

M CAROLINE HRUBETZ (Introduced by H B Williams)

*From the Department of Physiology, College of Physicians and Surgeons, Columbia University, New York City*

Bang<sup>1</sup> presented curves for the blood sugar level of rabbits after both intravenous and subcutaneous administration of epinephrine. The rise after intravenous injection was less than 100% and reached its maximum in  $\frac{1}{2}$  hour, that after subcutaneous injection around 300% and reached its maximum between the second and third hour after injection. One-tenth of a milligram was administered per animal.

In the present study, all injections were given subcutaneously. Abbott's "adrenalin" was diluted so that 1 cc contained 0.4 mg, making the full dose of 0.4 mg per kilo, or, 0.1 mg per 250 gm body weight. For the  $\frac{1}{2}$ ,  $\frac{1}{4}$ ,  $\frac{1}{8}$  doses corresponding dilutions were made. The animals used were normal-fed rats. We obtain curves similar to those of Bang except that our maximum rise occurred at the  $1\frac{1}{2}$  hour interval after injection. This discrepancy may be explained by our larger number of observations where variations of individual animals are smoothed out, and also, our smaller dosage.

Approximately 50 observations were made at each of the intervals 5, 15, 30, 45 minutes, 1,  $1\frac{1}{2}$ , 2 and 4 hours after the full dose and also after the  $\frac{1}{4}$  dose. In addition, 50 observations were made at the 30-minute interval for both  $\frac{1}{8}$  and  $\frac{1}{2}$  the full dose. Two series of controls of 50 observations each were made, the determinations of which were dispersed throughout the period of experimentation. The 0.2 cc Somogyi modification<sup>2</sup> of the Shaffer-Hartmann<sup>3</sup> blood sugar method was used.

There is a steady rise in the blood sugar level until the  $1\frac{1}{2}$ -hour interval is reached, the greatest change occurring during the first 20 minutes. After  $1\frac{1}{2}$  hours the blood sugar level is gradually reduced but does not reach the normal level in 4 hours. The mean deviations are smallest for the shortest intervals (6 to 11 mg for 5 minutes) and become progressively greater and more variable until the 4-hour interval is reached (17 to 46 mg). Eadie and Macleod<sup>4</sup> found it impossible to standardize insulin by the Epine-

<sup>1</sup> Bang, *Der Blutzucker*, Weisbaden, 1913, 113

<sup>2</sup> Somogyi, M., *J Biol Chem*, 1926, 70, 599

<sup>3</sup> Shaffer, A. P., and Hartmann, A. F., *J Biol Chem*, 1920, 45, 349

<sup>4</sup> Eadie, G. S., and Macleod, J. J. R., *Am J Physiol*, 1922, 46, 285

phrine Equivalent Method because of the great variability in the results. However, they took their blood samples at  $\frac{1}{2}$ , 1 and 2 hours after the injection, intervals where the mean deviations are the greatest.

The blood sugar level after  $\frac{1}{8}$ ,  $\frac{1}{4}$ ,  $\frac{1}{2}$ , and the full dose  $\frac{1}{2}$  hour after the injection shows a steady rise in the blood sugar until the  $\frac{1}{2}$ -dose is approached, after which the curve flattens out. Apparently, there is a maximum amount of stimulus to which the system will respond. Beyond this point, there is no increase in effects. The mean deviations vary from 13 to 29 mg, the smallest deviation occurring with the smallest dose.

*Summary* 1 1000 observations were made at 5, 15, 30, 45 minutes, 1,  $1\frac{1}{2}$ , 2 and 4 hours after given doses of epinephrine. 2 The blood sugar reaches its highest level in  $1\frac{1}{2}$  hours and has not returned to normal in 4 hours. 3 With doses varying from  $\frac{1}{8}$  to the full dose, the blood sugar increases proportionately with the dosage until  $\frac{1}{2}$  the dose where the curve flattens out. 4 The smallest deviations are obtained after the shortest interval after the injection, or, after the smallest dose.

## 7622 P

### Perimetry with Stimuli of Minimal Duration

LEO L. MAYER (Introduced by L. J. Pollock.)

*From the Departments of Ophthalmology, Nervous and Mental Disease, and Experimental Surgery of the Northwestern University Medical School*

The utility of field defects as outlined by perimetry in localizing disease along the optic pathways is fully discussed by Traquair<sup>1</sup> and Peter.<sup>2</sup> With the advent of the McHardy<sup>3</sup> self-registering perimeter and its electrical test objects a comparison between white and colored lights became available.

During the course of some experiments upon the chronaxia of the optic nerves projected by Davis and Pollock, a method of perimetry with a light stimulus of very short duration has been developed.

<sup>1</sup> Traquair, H. M., *Introduction to Clinical Perimetry*, American edition, St. Louis, C. V. Mosby Co., 1927.

<sup>2</sup> Peter, L. C., *Principles and Practices of Perimetry*, 2nd Edition, Philadelphia, Lea and Febiger, 1923.

<sup>3</sup> McHardy, M., *Ophth. Rev.*, 1882, 1, 107.

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Twenty cases were in this group. Most of the patients were epileptics in whom an organic basis was suspected.

In a large group with pituitary disease the flash method more critically emphasized the field defect. For example, where the ordinary perimetric study revealed a temporal field including the ninetieth meridian, the flash method recorded this temporal field to only the fiftieth meridian. Even meticulous studies with the Bjerrum method<sup>6</sup> did not approximate the accuracy shown by the flash method. Detailed studies of the 28 patients of this group will be published later.

Group 4 in which other intracranial lesions were investigated consists of a heterogeneous group with various lesions. Here the outstanding features of the flash method were the ease with which the patient was able to determine the critical point, the definiteness of the test target, and certain deviations from the usual findings in ordinary perimetry, such as bizarre configurations of the fields, etc. Six patients in this group demonstrated the dependability of the flash method in comparison with ordinary perimetry. Definite and characteristic field defects were demonstrable earlier and more accurately.

Of the last group in which intraocular disease was investigated, it appears that a closer check on the reduction of the field due to glaucoma may be had by this method. Also a better method of prognosticating in operated cases of detached retina is at hand. Only 2 cases of retinal detachment and 2 of glaucoma are included in this group as the study of ocular conditions, *per se*, has just started.

Many of the patients examined by this method have been operated, others have been autopsied, and confirmation of the flash findings were made. These will be reported later.

**Conclusions** 1 A method is described in which a rapid light flash is made use of as a perimetric target. 2 The technique of the examination is simple, neither the observer nor the patient become fatigued by the procedure and the perception of the flash being critical. The flash is recognized or it is not seen. Field defects are discovered to a degree not detectable by the older, far more painstaking method, which required greater cooperation and intelligence on the part of the patient. 3 Although a complete interpretation of all the findings delineated by this method must await further case study and subsequent confirmation by biopsied and autopsied materials, it appears that the method has certain advantages in localization of lesions of the optic pathways.

<sup>6</sup> Bjerrum, *Verhandl d X Internat Med Cong*, Berlin, 1890, 466, (II)

Our immediate problem was to compare the fields obtained by using a test object of varying size and color at infinite duration with that of a flash of light of a known and standard candle power and varying extremely short duration. Thus not only energy but time was considered a factor in our work. As the peripheral portion of the retina is stimulated there is a distinct varying limit to the recognition of light flashes of short duration, the more peripheralwards the stimulus, the longer must be its duration to be perceived. This has been amply confirmed by a previous study.<sup>4</sup> The work of Sheard<sup>5</sup> would seem to show that such a minute quantity as a quantum of light is enough to stimulate the tovea.

The instrument for producing such short flashes consists of a small neon bulb which is activated by condensor discharges through the use of "B" batteries. Flashes of the speed of  $1/25,000$  of a second were produced for this initial experiment. The neon bulb was blackened so that the light emitted occupied an area comparable to the ordinary 2 mm test object, and the bulb was mounted on a hand perimeter where it would be freely movable. A norm was established by recording the averages of 3 determinations on the eyes of each of 20 normal individuals. As the neon light is red in color it was necessary to determine whether the color itself was a factor. It was definitely shown that the field with the neon lamp did not correspond with the ordinary field for red.

The cases studied have been grouped as follows: (1) Normal fields in suspected intracranial disease, (2) Fields in hysteria, (3) Fields in pituitary disease, (4) Fields in other intra-cranial lesions, and (5) Fields in intraocular disease.

In the first group it was shown that the flash field afforded a more accurate indication than a form field or at times other localizing signs. For example in a patient with X-ray evidence of *sella turcica* destruction a normal flash field being found, operation failed to reveal any pathology interfering with the optic pathways. Ten cases were included in this group. In spite of the fact that ordinary perimetry with a white target was suggestive of a characteristic field defect in these cases, the examination by the flash method revealed normal fields. Subsequent examination of these cases confirmed the findings of the normal flash field.

The outstanding observation in the group of fields in hysteria was the fact that in no case was a tubular field demonstrated by the flash method although ordinary perimetry had shown this defect.

<sup>4</sup> Mayer, L. L., *Arch Ophth*, 1933, 9, 353

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<sup>5</sup> Sheard, C., *Am. J. Physiol. Optics*, 1922, 3, 126

*Protocol* One gm testis triturated and suspended in 10 cc saline After slight centrifugation, 80 cc of the uniform suspension were used for adsorption. Adsorption with buffered (pH 5.6) and unbuffered kaolin, left in icebox 24 hours The contents of each of the 2 tubes were eluted with (I) 4 cc N/100  $\text{NH}_4\text{OH}$ , or (II) 4 cc N/500  $\text{NH}_4\text{OH}$  Rabbits inoculated 11/23/33 Reactions recorded as —, +, ++, +++, according to area of induration

TABLE I

	Rabbit reaction*	Esbach	Ninhydrin
Supernatant fluid after adsorption			
(a) with buffer I	+	+	++++
(b) " " II	±	+	++++
(c) without buffer I	+	+	+++
(d) " " II	++	+	++++
Eluate I			
with buffer N/100 $\text{NH}_4\text{OH}$	+	+	++++
" " N/500	+	+	++++
without buffer N/100 $\text{NH}_4\text{OH}$	++	+	++++
" " N/500	++	+	++++
Eluate II			
with buffer N/100 $\text{NH}_4\text{OH}$	+++	±	++
" " N/500	+++	±	++
without buffer N/100 $\text{NH}_4\text{OH}$	+++	±	±
" " N/500	++	±	±
Eluate III			
with buffer N/100 $\text{NH}_4\text{OH}$	++	—	—
" " N/500	++	—	—
without buffer N/100 $\text{NH}_4\text{OH}$	++	—	—
" " N/500	++	—	—

\*Reading 4th day after inoculation

tions although it still contained active virus The second eluate contained the highest concentration of virus

*Experiment 2* This experiment was designed to ascertain the amount of virus eluted and the conditions most favorable for maximum elution The original material as well as the respective eluates were titrated on the rabbit skin To overcome the error due to variations in susceptibility of individual rabbits, a given dilution of each of the materials was inoculated into the same animal Each animal was thus inoculated on 6 separate points, 5 rabbits being used for the experiment This procedure also facilitated the comparison of the relative intensity of the reactions to different eluates, making the results as nearly quantitatively comparable as is possible in animal work Nitrogen determinations were made on all the materials tested \*

It will be noted that the kaolin adsorbs about 80% of the protein and nearly all the virus It is also of interest that the first eluate contains most protein and is weakest in virus, this is probably due to the neutralization of the  $\text{NH}_4\text{OH}$  by the buffer, thus

\*The nitrogen determinations were made by Dr Rosenberg

## Protein-Free Suspensions of Virus VI Purification of Vaccine Virus by Adsorption and Elution

I J KLIGLER

*From the Department of Hygiene and Bacteriology, Hebrew University, Jerusalem*

It was shown<sup>1</sup> that by repeated elution with N/500  $\text{NH}_4(\text{OH})$  from the same adsorbate it was possible to obtain a potent phage suspension which gave negative protein and ninhydrin tests and contained only 1.4 to 2.0 mg non-ammonia N per 100 cc of eluate.

In the present paper experiments are reported showing that it is possible with the same procedure to obtain an equally pure active suspension of vaccine virus.

*Technique* An infected rabbit testicle is removed on the 4th or 5th day after inoculation, ground in a mortar with sterile glass, and saline added slowly to give a 10% tissue suspension. After thorough trituration the tissue suspension is transferred to a centrifuge tube and centrifuged slightly to remove coarser particles. The material is handled aseptically to avoid contamination during the preparation. The heavy tissue suspension is then added to tubes containing 50% kaolin in saline, in the proportion of one part virus to one part kaolin suspension, thoroughly shaken, left in the icebox overnight and then treated in the manner previously described.<sup>2</sup>

The following experiments illustrate the details of the procedure employed and results obtained.

*Experiment 1* The suspension was prepared in saline and adsorbed in buffered and unbuffered saline suspensions of kaolin. The adsorption was carried out in duplicate and each adsorbate eluted separately. The supernatant fluids after the kaolin adsorption as well as the individual eluates from the adsorbate were tested on rabbits by the injection of 0.1 cc of the material intradermally. The results are summarized in Table I.

Adsorption with kaolin removed the greater part of the protein and virus from the suspension, and repeated elution with ammonia ultimately yielded a potent virus suspension which gave a negative protein reaction. The buffered kaolin adsorbed more completely but showed no difference in its response to elution. The first and second eluates still gave positive tests for protein and aminoacids, the third eluate, however, no longer gave positive Esbach and ninhydrin reac-

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<sup>1</sup> Kligler, I. J., and Ohitzki, L., *Brit J Exp Path*, 1934, 13, 14.

*Protocol* One gm testis triturated and suspended in 10 cc saline. After slight centrifugation, 8.0 cc of the uniform suspension were used for adsorption. Adsorption with buffered (pH 5.0) and unbuffered kaolin, left in boxes 24 hours. The contents of each of the 2 tubes were eluted with (I) 4 cc N/100  $\text{NH}_4\text{OH}$ , or (II) 4 cc N/500  $\text{NH}_4\text{OH}$ . Rabbits inoculated 11/23/33. Reactions recorded as —, +, ++, +++, according to area of induration.

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" " N/500	++	+	+++
without buffer N/100 $\text{NH}_4\text{OH}$	++	+	+++
" " N/500	++	+	+++
Eluate II	+++	±	++
with buffer N/100 $\text{NH}_4\text{OH}$	+++	±	++
" " N/500	+++	±	++
without buffer N/100 $\text{NH}_4\text{OH}$	+++	±	++
" " N/500	+++	±	++
Eluate III	++	—	—
with buffer N/100 $\text{NH}_4\text{OH}$	++	—	—
" " N/500	++	—	—
without buffer N/100 $\text{NH}_4\text{OH}$	++	—	—
" " N/500	++	—	—

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tions although it still contained active virus. The second eluate contained the highest concentration of virus.

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<sup>1</sup> Kligler, I. J., and Oltzki, L., *Brit J Exp Path*, 1934, 15, 14.



**Protocol** Infected testes were triturated and a 10% suspension made in saline. The adsorption was made as above by mixing equal portions of the suspension with buffered kaolin. The elution was carried out with N/200  $\text{NH}_4\text{OH}$ . Four successive elutions were made. The eluates as well as the original suspension were titrated on the rabbit skin. The readings were made on the 4th and 5th day after inoculation. The table gives the results at the last reading.

TABLE III

Virus Dilutions	Original Suspension	Eluates			
		I	II	III	IV
10,000	+++	++	++	++	++
100,000	+++	+	++	++	++
1,000,000	+++	+	+	+	+
10,000,000	+++	+	+	+	+
Esbach reaction	+++	+	+	+	+
Ninhydrin	+++	++	+	+	+

The signs  $\pm$ , +, ++, etc., indicate intensity of reaction, tr = trace

of virus by increasing tissue permeability thus causing a more extensive lesion. Normal testicular extract added to the eluates increases the extent and severity of the lesion.

**Summary** Experiments are presented showing that it is possible by adsorption with kaolin and subsequent successive elutions with ammonia to obtain a potent suspension of vaccinia virus giving negative Esbach and ninhydrin tests and containing 10 to 27 mg non-ammonia N per 100 cc of fluid. The severity of the reaction produced by a given dilution of the original testicular suspension is always greater than that produced by an equal volume of the same dilution of the eluted pure virus. In most instances the eluates were active in as high a dilution as the original suspension. The vaccinating efficiency of the pure virus has not yet been tested but it is anticipated that it may prove useful for intradermal vaccination particularly because of the milder character of the reaction produced by the purified virus.

Protocol, Exp 2 Material not centrifuged before adsorption. Tissue emulsion 1/10 mixed with equal amounts of kaolin suspension. All elutions made with N/500 NH<sub>4</sub>OH B = with buffer, W = without buffer

TABLE II

	Supernatant fluid			Eluates (undiluted)							
	Original material		after adsorption	I		II		III		IV	
				B	W	B	W	B	W	B	W
	(a) Test of Virus										
Rabbit Test*	+++	++	++	++	+++	+++	+++	+++	+++	++	++
Non NH <sub>3</sub> N (mg %)	280	458	334	512	91	472	631	395	544	268	104
Esbich Reaction	+++	++	++	+	+	±	±	tr	tr	—	—
Ninhydrin Reaction	+++	++	++	+	+	±	±	±	±	—	—

(b) Titration of Virus

	Dilution tested	Original suspension	Supernatant Fluid	Eluates			
				I	II	III	IV
Rabbit 1	1 100	+++	±	+	+	+	±
" 2	1 1,000	++	±	+	++	++	++
" 3	1 10,000	+++	—	±	+	++	++
" 4	1 100,000	++	—	±	+	+	±
" 5	1 1,000,000	+	—	—	±	+	±

\* —, +, ++, +++ indicate relative intensity of the reaction. The rabbit results are the readings on the 5th day after inoculation.

reducing its eluting capacity. The third eluate was the most potent, but the fourth eluate was still active in a 1 1,000,000 dilution, the highest dilution tested. The protocol also brings out clearly the differences in the reactivity of animals, the 1 1,000 dilutions producing milder lesions than the 1 10,000.

*Experiment 3* The previous experiment was repeated with the purpose of carrying the dilutions to the maximum and comparing the virus content in the respective eluates with that in the original suspension. The procedure was the same as before. The results are summarized in Table III.

The titration experiments yielded paradoxical results. The eluates were active in as high a dilution as the original tissue emulsion, at the same time, in the lower dilutions, the reactions produced by the tissue suspension were more severe than those produced by the eluates. The greater severity of reaction produced by the original tissue suspension is ascribable to the phenomenon observed by Duran-Reynals<sup>2</sup>. The testicular extract increases the invasiveness

<sup>2</sup> Duran-Reynals, F., *J. Exp. Med.*, 1929, 50, 327

A preliminary rough experiment, made August 20th, 1934, on a 25% suspension of normal yeast showed that the time of reduction of cytochrome was greatly accelerated by DNC, the time required, after aeration, for reappearance of the d band being reduced from 27 seconds to 5 seconds. After repeated confirmation of these results experiments were conducted on starved yeast with DNC in the presence of a variety of substrates.

Typical results of such experiments on starved yeast are presented in Tables I and II. The procedure in each case was as follows. A 3 cc portion of a well aerated 17% suspension of Fleischmann's yeast in pH 6 McIlvaine phosphate-citrate buffer was mixed with 1 cc portions of the designated substrate, DNC, and buffer solutions to give a constant volume of 5 cc. The final concentrations were Yeast 10%, substrate, 0.2%, DNC,  $5 \times 10^{-4} M$  for the experiments in Table I, and  $6.25 \times 10^{-4} M$  for the experiments in Table II. After 10 minutes reaeration, the effect was registered by noting the number of seconds which elapsed prior to the reappearance of the strong d band. Since the degree of acceleration effected by the DNC varies according to the length of time that the suspension has been allowed to stand, controls with yeast and buffer alone and yeast and DNC and buffer were run at frequent intervals in the course of the experiments.

TABLE I.

	Cysteine		Succinate		Glucose		Pyruvate		Lactate	
	None	0.2%	None	0.2%	None	0.2%	None	0.2%	None	0.2%
No DNC	292	272	285	243	223	63	164	65	106	93
$5 \times 10^{-4} M$ DNC	66	58	62	53	63	27	61	56	45	49

TABLE II.

	Cysteine		Succinate		Glucose		Pyruvate		Lactate	
	None	0.2%	None	0.2%	None	0.2%	None	0.2%	None	0.2%
No DNC	422	303	346	297	254	89	194	104	118	96
$6.25 \times 10^{-5} M$ DNC	90	85	89	100	91	50	88	94	86	83

The data show (a) that DNC greatly accelerates the reduction of cytochrome and (b) that only with glucose is the reducing ability of the combination equal to the sum of those of the individual agents. Indeed, the pyruvate plus DNC and lactate plus DNC are no more efficient than DNC alone, although pyruvate and lactate both have a large reducing effect when used singly. It may be noted that this inability of lactate to increase the reduction of cytochrome in the

# Observations on Cellular Oxidative Mechanisms Involved in Dinitrophenol Stimulation of Respiration

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In an extensive investigation of the effect of dinitro compounds on tissue respiration and cell division, a point has been reached where it appears desirable to know more about the mechanism by which such compounds stimulate oxidative processes in the cell. We report here some experiments with 4,6 dinitro-o-cresol (DNC)\* which indicate that dinitro compounds do not act on cell respiration either in the same way as methylene blue and other dyes which are reduced by the cell and re-oxidized by molecular oxygen<sup>1</sup> or in the same way as dimethyl-p-phenylene diamine, which is reduced by the cell and reoxidized by the indophenol oxidase.

When the oxygen consumption of sea urchin eggs is raised by DNC to 400% of the normal,<sup>2</sup> the R Q remains unchanged at the normal value of about 0.93. We have found that DNC is not an autooxidizable catalyst for the oxidation of glucose or cysteine, even in the presence of traces of metals or cytolyzed animal tissue. We have also found with rat tissues and eggs of invertebrate marine animals that the action of DNC as a respiratory stimulant can be completely and reversibly blocked by cyanide. Field, Martin and Field report similar results with cyanide on yeast.<sup>4</sup>

These facts indicate that DNC acts upon one or more of the cyanide sensitive oxidative chains in the cell. These may be roughly divided into 2 classes: (A) those depending for oxygen activation on Keilin's cyanide sensitive indophenol oxidase, and (B) those in which the substrate is activated by an autooxidizable cyanide sensitive dehydrogenase.<sup>5</sup> The mechanisms classifiable under (B) do not act through cytochrome, hence a distinction between the 2 classes can be made by studying the effect of DNC on the rate of oxidation or reduction of cytochrome in respiring cells.

\* This compound is 1-methyl-2-hydroxy-3,5-dinitrobenzene.

<sup>1</sup> Barron, E. S. G., *J. Biol. Chem.*, 1929, **81**, 445.

<sup>2</sup> Keilin, D., *Proc. Roy. Soc. (London)*, B, 1929, **104**, 206; Runnström, J., *Protoplasma*, 1932, **15**, 532.

<sup>3</sup> Krahl, M. E., and Clowes, G. H. A., *Biol. Bull.*, 1934, **67**, 332.

<sup>4</sup> Field, J., 2nd, Martin, A. W., and Field, S. M., *Proc. Soc. Exp. Biol. and Med.*, 1934, **31**, 997.

<sup>5</sup> Dixon, M., *Biol. Rev.*, 1920, **4**, 352.

Harvey,<sup>1</sup> using a cholecystnephrostomy, produced ulcer in 17 out of 43 animals. Bollman and Mann<sup>2</sup> by simply ligating the common ducts produced ulceration in about 60%. In the experiments of Berg and Jobling<sup>3</sup> in which a Rous type of fistula was used, ulcers resulted in 13 of 23. Kim and Ivy report 10% ulcers in one series<sup>4</sup> in which the common duct was tied, but 60% in cases of Rous fistula.

Besides the obvious acid factor, there is considerable evidence that other influences are at work. Ivy has suggested that the mechanical irritation of the tubing near the duodenum may be important. On the other hand Elman has seen but few ulcers resulting from bile fistula alone. Our own experience has been that simple duct ligation alone caused ulceration in numbers about one-half the rate usually suggested (60%), but that a very high percentage resulted from the Rous fistula which has proven very difficult technically in our hands. Berg suggests that the general condition of the dog is very important and found that animals kept under poor hygienic conditions developed ulcer in 100% while those better cared for showed only 30%.

Using the Dragstedt cannula for making the biliary fistulas we have been surprised at the freedom from this complication. Twenty such experiments have been made and not a single ulcer has developed, as evidenced either by post-mortem examination or presence of blood in the stools. These animals have been fed on various diets, some with large amounts of meat and others with high carbohydrate content. Some have undergone periods of starvation and several had one or more exploratory laparotomies. All had total fistulas in which no licking of bile occurred and all lost weight progressively. Several had lost as much as a third of body weight. Some lived over 5 months.

These experiences suggest that the general condition of the animal is not the deciding factor. It would be hard to find animals more cachectic than ours. Diet is evidently not important. In view of the fact that previously our bile fistula dogs prepared by cholecystnephrostomy or by Rous fistulas constantly developed ulcers, and that the incidence of ulcer among duct ligation dogs was high, it seems suggestive that the only common factor is concomitant liver damage. In all the above types of operations, in our hands at least there has been a progressive development of degenerative and in-

<sup>1</sup> Kapsin, R., Eagle, L. P., and Harvey, S. C., *S. G. O.*, 1924, **39**, 65.

<sup>2</sup> Bollman, and Mann, F. C., *Arch. Surg.*, 1932, **24**, 126.

<sup>3</sup> Berg, B., and Jobling, J. W., *Arch. Surg.*, 1930, **20**, 997.

<sup>4</sup> Ivy, A. C., and Culev, L. N., *Am. J. Surg.*, 1931, **11**, 531.

presence of DNC may account for the aerobic glycolysis which dinitro compounds produce in tissues<sup>6</sup>

In subsequent experiments with iodoacetate poisoned yeast, carried out in the manner described above with M/1500 iodoacetate in the pH 6 buffer, it was found that DNC gave no acceleration of cytochrome reduction even when lactate or glucose was present. Since others<sup>7</sup> have observed that iodoacetate blocks the acceleration by 2,4 dinitro phenol of respiration in yeast, it is likely that some sulfhydryl containing enzyme system is essential for the DNC action<sup>8</sup>

We have also found that such non-specific dehydrase poisons as sodium pyrophosphate and narcotics inhibit to a limited degree the reactivity of tissues to DNC stimulation

From the evidence available we believe it likely that DNC stimulates cellular respiration by accelerating the oxidation by cytochrome of some substrate previously or simultaneously acted upon by the anaerobic dehydrases of the cell. It is too early to say whether DNC acts as a diffusible oxygen carrier between cytochrome and the substances normally oxidized or as an artificial substitute for a co-enzyme in the activation of substrates which do not normally play an important rôle in respiration. We are continuing these studies with cell-free cytochrome and individual dehydrases in an effort to demonstrate the relation between the individual components of such a system, eliminating at the same time the complicating factors connected with variations in the permeability of the cell

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#### Studies on Acholic Cachexia IV Relation of Biliary Diversion to Duodenal Ulcer Formation \*

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The occurrence of peptic ulcer of the duodenum after the exclusion of bile has been noted with a wide variation in frequency and the factors concerned are not clearly understood. Kapsinow and

<sup>6</sup> Dodds, E. C., and Greville, G. D., *Nature*, 1933, **132**, 966, Dodds, E. C., and Greville, G. D., *Lancet*, 1934, **1**, 398.

<sup>7</sup> Ehrenfest, E., and Ronzoni, E., *Proc. Soc. Exp. Biol. and Med.*, 1933, **31**, 318.

<sup>8</sup> Dickens, F., *Biochem. J.*, 1933, **29**, 1141, Michaelis, L., and Schubert, M. P., *J. Biol. Chem.*, 1934, **106**, 331.

\*Work done in part under a grant from the Jessie Horton Koesler Fellowship

about one-half hour before the first or control electrocardiogram. Light ether anesthesia followed, then rib resection just to the left of the sternum to expose the pericardium, followed by artificial respiration and opening the pericardial sac by a 3 to 5 cm anterior incision. There was an electrocardiographic control at each step. The pericardium and chest were not closed again.

The chemicals used were liquids, solutions, and solids (crystals, powders), the fluids in amounts averaging one cubic centimeter applied at body temperature with a medicine dropper or camel's hair brush so that pressure effects were absent. The solids were dropped on lightly. The initial application was made over the left ventricle just to the left of the septum and above the apex. Subsequent spreading of fluids was unavoidable.

The experiment was stopped if the dog went into shock, became asphyxiated, or developed ventricular fibrillation.

*Electrocardiographic Results Controls* These proved to be very important. The ST takeoff varied from +2 mm to -1 mm and in lead II half the T waves were negative or diphasic. Ether anesthesia caused surprising transient voltage decrease, as well as rate increase, and opening the pericardial cavity to atmospheric pressure and room temperature caused another voltage decrease (all leads) which did not tend to return to the control level.

Furthermore, the control ST segment was decidedly convex or concave in some cases, and about half the time, ST or T changed direction in at least one lead after ether anesthesia.

TABLE I  
Substances Producing ST Elevation as the Chief Result

Alcohol (95%)	ST rises starting in 12 seconds, with return to normal in 1 minute. An even greater rise occurred with reapplication over an artery.
Calcium Chloride (7% 10% sol)	Marked ST rises within one half minute, maximal in 3 minutes, then partial return to normal. (Concentrations of less than 7% were ineffective.)
Ether (U.S.P.)	Marked sudden ST rise with sharp T inversion.
Ethyl Chloride (U.S.P.)	ST elevation and deep T waves.
Formic Acid (U.S.P.)	ST rise in 10 seconds, starting towards normal in 1 minute.
Hydrogen Peroxide (U.S.P.)	Transient ST and T change more marked when application was over an artery. Voltage decrease.
Lactic Acid (1%)	Instantaneous ST elevation.
" " (Conc.)	Rapid but not instantaneous ST elevation.
Mercurochrome (Crystals)	ST elevations starting in 6 minutes, maximum in 15 minutes.
Potassium Chloride (1% 10% sol)	ST rises similar to those from calcium chloride, and agreeing with Wigger's results. <sup>8</sup>
Sodium Chloride (Crystals)	Moderate ST elevation.
Tincture of Iodine (U.S.P.)	Similar to alcohol. (Tincture of iodine contains 83% alcohol.)

fective changes in the liver parenchyma. In the successful type of Rous fistula (ours were usually failures) Elman found very few ulcers. Berg's experience was similar. His smoothly functioning Rous fistulas developed many ulcers while the unsuccessful ones developed many.

It appears, therefore, that the development of duodenal ulcer in the absence of bile from the intestine is partially dependent on some factor other than acid. Possibly the effect on the gastric motility of liver damage, as suggested by Still and Carlson,<sup>7</sup> is the deciding factor.

## 7626 C

## Electrocardiographic Studies of Chemical Pericardial Irritation

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From the Lasker Foundation for Medical Research and the Department of Medicine of the University of Chicago

Pericardial effusions, clinical or experimental, may result in electrocardiographic changes much like those of coronary occlusion or of ligation of the coronary arteries.<sup>1-3</sup> In experimental effusions, one must consider the effect of pressure and of possible chemical influence. Indeed, Wiggers<sup>4</sup> has demonstrated bizarre ventricular complexes, similar to those of effusions, resulting from applying a few cubic centimeters of potassium chloride solution to the visceral pericardium. Hence it seemed desirable to study the effects of various bland and irritating chemicals under conditions to exclude any pressure factor.

Dogs were used, with preliminary morphine sulphate 0.015 gm

<sup>1</sup> Kim, M. S., and Ivy, A. C., *J. A. M. A.*, 1931, **47**, 1511.

<sup>2</sup> Elman, R., and Hartmann, A. F., *Arch. Surg.*, 1931, **23**, 1030.

<sup>3</sup> Still, K. S., and Carlson, A. J., *Am. J. Phys.*, 1929, **80**, 34.

\* I am deeply indebted to Dr. Louis Leiter and Dr. Emmet Bay for advice during this work.

<sup>1</sup> Smith, F., *Arch. Int. Med.*, 1918, **22**, 8.

<sup>2</sup> Smith, F., *Arch. Int. Med.*, 1920, **23**, 673.

<sup>3</sup> Smith, F., *Arch. Int. Med.*, 1923, **32**, 497.

<sup>4</sup> Barnes, A. R., and Mann, F. C., *Am. Heart J.*, 1932, **7**, 477.

<sup>5</sup> Scott, R., Feil, H., and Katz, L., *Am. Heart J.*, 1929, **5**, 68, 77.

<sup>6</sup> Bay, E. B., Gordon, W., Adams, W., *Am. Heart J.* 1933, **8**, 525.

<sup>7</sup> Harvey, J., and Scott, J. W., *Am. Heart J.*, 1932, **7**, 532.

<sup>8</sup> Wiggers, C., *Am. Heart J.*, 1930, **5**, 346.



TABLE II  
Substances Producing Complete Bundle Branch Block.

Oil of Cloves (U.S.P.)	Complete in 1 hour
Sandalwood Oil (U.S.P.)	" " 28 seconds
Turpentine (U.S.P.)	" " 9 minutes

(In each of these instances, the PR interval was unaltered, the evolution of the block was gradual, with no transitional period resembling arborization block.)

TABLE III  
Substances Producing Miscellaneous Results

Ammonia Water (1% 10% U.S.P.)	Occasional extrasystoles and slight ST changes
Barium Chloride (1.5% sol.)	Varying runs of extrasystoles
Chloroform (U.S.P.)	Sudden, sharp, diphasic T wave
Potassium Permanganate (0.1% sol.)	Slight QRS notching, diphasic T, and high P
Silver Nitrate (20% sol.)	Temporary QRS widening, but after bilateral vagotomy extrasystoles occurred, and after atropine, nothing happened, except the slower rate
Sodium Carbonate (U.S.P.)	Transient ventricular tachycardia of tremendous voltage. Later, ST and T high. Still later, return to normal
Sodium Hydroxide (10% sol.)	Bizarre notchings of uncertain significance (Possibly respiratory variations)

(see lactic acid record on Plate 1) to an hour or more. The changes with alcohol, calcium chloride solution, lactic acid, formic acid, sandalwood oil, and sodium carbonate crystals were some of those occurring within one minute.

It is interesting to note the variety of substances that were without significant effect (Table IV).

TABLE IV  
Substances Producing No Significant Result

Acetone (U.S.P.)	Normal Saline
Chrysarobin (crystals)	Potassium Chlorate (5% sol.)
Ephedrin Sulphate (3% sol.)	Talc (U.S.P.)
Glucose (U.S.P.)	Tannic Acid (5% sol.)
Lactose (U.S.P.)	Xylol (C.P.)

*End Effects* Some of the changes returned toward normal, others progressed into ventricular fibrillation, or were not observed. After several hours, it was sometimes difficult to tell whether the effects observed were due to the original application, shock, or asphyxia.

*Preliminary Atropinization or Bilateral Vagotomy* These procedures gave important variations from the usual results in some cases. (The average dose of atropine was 0.36 mg per kilo intravenously.) Thus, alcohol caused a marked ST rise which failed to occur after atropine. Silver nitrate, which caused temporary widening of the QRS, resulted in extrasystoles after bilateral vagot-

In a few instances, for control purposes, nothing was done for several hours after opening the pericardium, there were no additional electrocardiographic variations. In about half the experiments the chemical was applied first to the intact parietal pericardium; there was never any significant change. Incidentally, lead II alone usually gave all the necessary information.

*Application to the Visceral Pericardium* The most frequent change was a genuinely significant rise in the ST interval, amounting at times to RT fusion, and substances quite different chemically produced similar results (Table I and Plate 1). Complete bundle-branch block occurred 3 times, in each case with similar substances (Table II).

There was a group giving miscellaneous results (Table III). The time of appearance of the changes varied from instantaneous

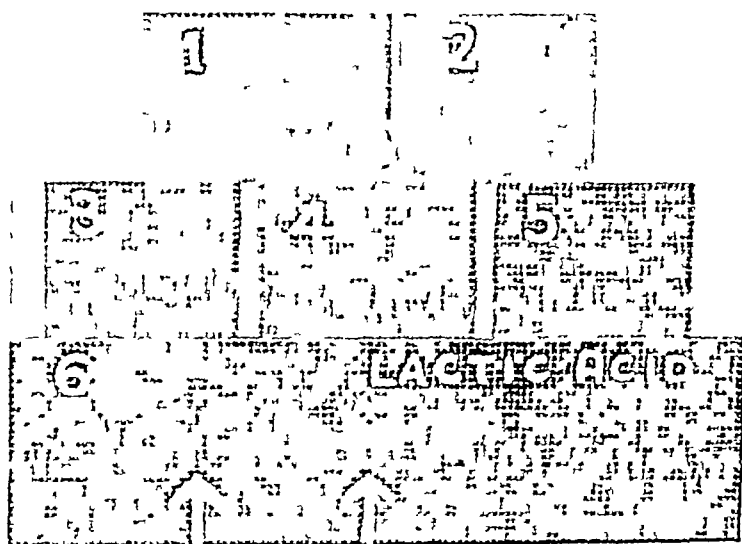


PLATE I  
(Lead II throughout)

FIG 1, 2 Calcium Chloride

Fig 1—30 seconds after applying 7% calcium chloride sol  
Fig 2—4 minutes " " " " " "

FIG 3, 5 Alcohol

Fig 3—12 seconds after applying 95% alcohol to the muscle  
Fig 4—60 " " " " " "

Note return toward normal

Fig 5—5 seconds after re application over branch of left coronary artery

FIG 6 Lactic Acid

Fig 6—Arrows indicate moment of application of lactic acid, 1% sol  
Note the immediate ST plateau like rise

bland and irritating chemicals to the visceral pericardium, with pressure factors absent, are described. The chief of these are marked ST elevations of several types, occurring quickly, bundle branch block, and the rarity of extrasystoles. The controls proved very important, and are emphasized.

## 7627 C

## Effect of Maximal Feeding on Metamorphosis in Amblystoma.

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Twitty and Schwind<sup>1</sup> have shown that in heteroplastic grafting between the 2 species *Amblystoma tigrinum* and *A. punctatum*, comparable results with respect to growth are obtained only when the hosts are all maintained at the same nutritional level, an effect obtained by maximal feeding, thus insuring the rapidly growing voracious *tigrinum* larvae opportunity to realize their full capacity for growth. The effect of maximal feeding upon the more slowly growing species has not been emphasized, although various workers have noted that along with the spectacular acceleration of the growth rate, and a consequently earlier metamorphosis, there is a high mortality during the metamorphic period in maximally fed groups of *A. punctatum*, regardless of the type of diet.

The present experiments were undertaken in order to show the results of quantitative variation in a single diet upon the developmental rate and viability of *A. punctatum* from the earliest feeding stage through metamorphosis. Two hundred animals, taken from several bunches of eggs all at approximately the same stage of development, were reared from stage 39 onward in separate finger bowls. At the feeding stage the animals were divided into 4 groups of 50 each. The first 50 animals were starved, the second group given one feeding weekly, the third fed 3 times a week, and the fourth fed maximally. *Enchytraeus*, a small white worm, was used as the sole diet. All animals were measured at approximately 2-week intervals, the total length being recorded.

Fig. 1 shows the average growth rate for each group. The minimally fed larvae ate poorly, had low vitality, and remained of ap-

<sup>1</sup> Twitty, V. C., and Schwind, J. L., *J. Exp. Zool.*, 1931, **50**, 61; Twitty, V. C., and Elliott, H. A., *J. Exp. Zool.*, 1934, **68**, 247.

omy, and in no change at all after preliminary atropine. However, the typical calcium chloride results were not prevented by atropine. Unfortunately, the experiments in which the volatile oils (with their bundle branch block type of curve) were used after preliminary atropine, gave unreliable results.

*Postmortem Observations* Thirty-four hearts were autopsied. None showed gross myocardial lesions, nor gross pericarditis. Microscopic sections were made under and adjacent to the irritated areas in 9 dogs (in the experiments using formic acid, hydrogen peroxide, potassium permanganate sol, sandalwood oil, sodium hydroxide sol, turpentine ammonia water, mercurochrome crystals, and tincture of iodine). In the last 3 of these, fragmentation and loss of striation were taken as evidence of a muscle injury to a depth of 0.1 to 0.3 mm. Otherwise there was no apparent muscle damage, pericardial injury was variable but slight.

*Significance of the Changes* Can one explain the chief changes ST elevation of various types, and 3 instances of bundle branch block? The experiments do not furnish proof but they do show that demonstrable muscle injury need not be present to produce the changes described and suggest that the extracardiac nerves may be a factor. Of course the failure to find histologic evidence of muscle injury does not preclude the possibility that the observed effect of certain substances (e.g. salts) depends upon absorption, and passage of ions between muscle fibers. Moreover, bundle branch block need not depend upon absorption. There have been fairly numerous reports of transient bundle branch block, wherein there was held to be no heart damage with increased vagal tone the factor responsible.<sup>9-13</sup>

The question of local or transient ischemia is not within this discussion. It is certainly questionable that any ischemia occurred in the experiments here reported. We note that alcohol or hydrogen peroxide applied over a coronary artery gave results more marked than over the muscle alone. One possibility is that the arterial application produced constriction and ischemia, but it is just as reasonable to assume increased reflex effects if the nerve supply is richer along the arterial course.

*Summary* The electrocardiographic results of applying various

<sup>9</sup> Wolff, L., Parkinson, J., and White, P. D., *Am Heart J*, 1930, **5**, 685

<sup>10</sup> Faulkner, J. M., *Med Clin of N Am*, 1932, **15**, 997

<sup>11</sup> Newman, M., *Br Med J*, 1931, **2**, 1134

<sup>12</sup> Carr, F. B., *New Eng J Med*, 1933, **200**, 1101

<sup>13</sup> Morris, R. S., and McGuire, J., *Am J Med Sci*, 1932, **184**, 202

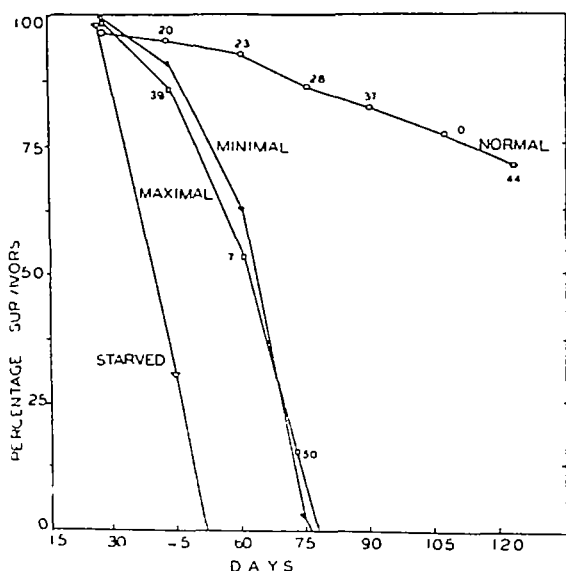


Fig 2  
Death rate in *A. punctatum*

had reached 50 millimeters. This is the length at which metamorphosis usually occurs, and 35% of the animals dying at this time were almost completely metamorphosed, with the gills reduced to short stumps and the color pattern changed. No maximally fed animals were brought through metamorphosis, although in the normal group over 50% of the animals metamorphosed normally at about the 120th day.

This high mortality at metamorphosis in maximally fed *A. punctatum* is in complete agreement with the work of Twitty (personal communication) who has used this method of feeding for several seasons and with larvae from different localities. It may also explain the failure of Patch<sup>3</sup> to obtain metamorphosis in *A. punctatum* fed on *Enchytraeus*. These results indicate that maximal feeding is not practicable in this species except in the earlier larval stages.

<sup>3</sup> Patch, E. M., *Proc Soc Exp Biol and Med*, 1927, **24**, 218

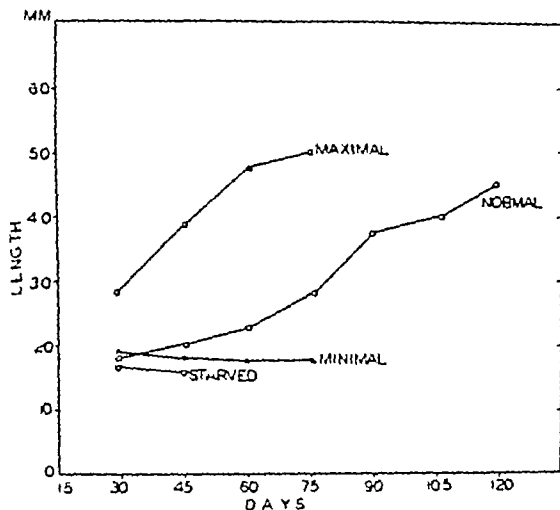


FIG 1  
Growth rate in *A. punctatum*

proximately the same size. By the 75th day most of these animals had died, before the 90th day the group was extinct. However, these animals survived a month longer than the starved group, all of which died by the 45th day. Three feedings a week—marked as “normal” because they approximate the usual laboratory feeding schedule—were enough to maintain a growth rate roughly about one-half that of the maximally fed group. The growth of the latter group approaches that of Twitty’s maximally fed animals in both rapidity and ultimate size attained, the largest specimen was 57 millimeters long by the 60th day, as compared with 46 millimeters reached by the largest normally fed animal by the 120th day. The average final size of the maximally fed animals is no greater than that reached by the same species in nature (Dempster<sup>2</sup>) but the growth rate is much more rapid and metamorphosis is earlier than in the tree-living specimens.

Correlated with the marked acceleration of the growth rate by maximal feeding was a high death rate, particularly in late larval stages. Early larval deaths were apparently no more frequent in one group than in another. In Fig 2 the percentage of survivors is plotted against time in days and the average body length in millimeters is indicated in small figures for the maximally and normally fed groups. The high mortality in the maximally fed group began after the animals had reached an average length of 40 millimeters with only 15% surviving by the time the group average

<sup>2</sup> Dempster, W. T., *Biol. Bull.*, 1930, 57, 182

that tasting of injected substances does not presuppose excretion through the saliva<sup>6</sup>

Other observations indicative of central action may be summarized briefly. In dogs, oral doses of 100 mg/kg of ethyl chaulmoograte cause emesis in from 35 to 205 minutes, allowing time for sufficient absorption of chaulmoograte, vomiting does not relieve the nausea, and should the vomitus be re-eaten by the dog some time later, emesis does not then recur. Na chaulmoograte in doses of 67 mg/kg causes emesis in dogs in an average time of 120 minutes although gastric irritation must be considerably greater than with either ethyl chaulmoograte or chaulmoogra oil. Dogs given emetic doses of chaulmoogrates every third day respond by vomiting more readily, conditioning of the response was ruled out by giving an equal dose of olive oil in gelatine capsules as a control. In a dog vomiting within 35 minutes after an oral dose of 100 mg/kg of ethyl chaulmoograte, 0.01 mg/kg of atropine (sufficient to antagonize quantitatively one minimal emetic dose of pilocarpine) delayed emesis to 85 minutes. Two-tenths mg/kg of atropine delayed emesis to 180 minutes, and if 0.2 mg/kg of atropine were given with 100 mg/kg of ethyl chaulmoograte and the same dose of atropine repeated once at 20, 40 or 80 minutes no emesis occurs. For cats the findings were essentially the same, oral doses of 75 mg/kg of Na chaulmoograte brought about emesis in an average time of 83 minutes although administration of 100 mg/kg of ethyl chaulmoograte produced extreme nausea but did not result in emesis in 7 of 9 trials with 6 cats even when given on the day following administration of the same dose. Two of 9 cats so treated vomited in 65 and 87 minutes. One hundred and fifty mg/kg of ethyl chaulmoograte produced emesis within an average time of 92 minutes in 6 of 6 cats. Intramuscular administration of the standard dose<sup>9</sup> of nicotine abolished the emetic response to ethyl chaulmoograte. Again, with dogs an emetic dose of morphine given one hour prior to 100 mg/kg of ethyl chaulmoograte prevented emesis, while an emetic dose of apomorphine, which does not have a secondary depressing effect on the vomiting center as does morphine,<sup>7</sup> given similarly did not prevent the emetic response.

<sup>6</sup> Winternitz, M., Deutsch, J., and Burell, A., *Mediz. Klinik*, 1931, **27**, 986, 1932, **28**, 831, Tarr, L., Oppenheimer, B. S., and Sager, R. V., *Am. Heart J.*, 1933, **8**, 766.

<sup>9</sup> Hatcher, R. A., and French, B. S., *J. Pharmacol. and Exp. Therap.*, 1932, **46**, 97.

<sup>7</sup> Leake, C. D., *J. Pharmacol. and Exp. Therap.*, 1922, **20**, 359.

## Mechanism of the Emetic Action of the Chaulmoogrates \*

GEORGE A. EMERSON (Introduced by C D Lenke)

*From the Pharmacological Laboratory, University of California Medical School, San Francisco*

Read<sup>1</sup> recently has cast doubt upon the finality of evidence previously advanced by himself<sup>2</sup> and others<sup>3</sup> as to the central emetic action of chaulmoogrates. The possibility of sufficient salivary secretion of chaulmoogrates to bring about local gastro-enteric irritation and reflex emesis negates conclusions drawn from observed effects of parenterally administered chaulmoogrates. It thus appears desirable to establish by criteria other than taste alone whether or not chaulmoogrates are present in saliva following therapy and also to study the nature of the emetic effect further.

Absence of rapid racemization of d-chaulmoogric acid in the body has been shown by Walker, MacArthur and Sweeney<sup>4</sup>. In the present experiments no traces of d-chaulmoogric acid were found polariscopically in ether-alcohol extracts of 5 to 10 cc acidified saliva samples of dogs, cats and a human volunteer taken after oral administration of gelatine capsules of chaulmoogric acid in doses of 10 to 100 mg/kg as the strongly dextrorotatory Na salt or ethyl ester. Concentrations to be effective *in vivo* in producing emesis would presumably exceed the 0.001 to 0.01 M solutions of Na chaulmoograte we have found active in stimulating isolated rabbit duodenum. Assuming a daily flow of 1400 cc of saliva for a human, approximately one-fifth of the administered dose of 10 mg/kg must then be excreted by the salivary glands to maintain a concentration of 0.01 M Na chaulmoograte in the saliva for one hour, which in itself appears improbable. Further, it is generally accepted

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\* Part of a cooperative study of the chemotherapy of leprosy conducted by the Pacific Institute of Tropical Medicine within the Hooper Foundation for Medical Research, and the Pharmacological Laboratory of the University of California Medical School, San Francisco.

<sup>1</sup> Read, B. E., *Internat J Lep*, 1933, **1**, 293.

<sup>2</sup> Reid, B. E., *J Pharmacol and Exp Therap*, 1924, **24**, 221.

<sup>3</sup> Lissner, H. H., *Am Rev Tuberc*, 1923, **7**, 257; Schwarz, L., *Z nahr Genussm*, 1911, **22**, 441; Valenti, A., *Archiv di Farmacol sper e Sci. off*, 1917, **24**, 23.

<sup>4</sup> Walker, E. L., MacArthur, C. G., and Sweeney, M. A., *Trans Nat Tuberc Soc*, 1923, 553.



adrenalin To date we have been able to demonstrate sphincter relaxation with so small a dose as 1-400,000,000 and a sphincter contracture with the minute dose of 1-1,000,000,000 of histamine We suggest the study of the iris sphincter strip as a probable tissue for biological assay as well as its adoption in pharmacological laboratory teaching

## 7630 C

## Pharmacology of Inflammation III Influence of Hygroscopic Agents on Irritation from Cigarette Smoke \*

MICHAEL G MULINOS AND RAYMOND L OSBORNE

*From the Department of Pharmacology, College of Physicians and Surgeons, Columbia University*

We herewith report a successful attempt to measure objectively the irritant properties of cigarette smoke We used the conjunctival sac of rabbits according to the technic of Hirschhorn and Mulinos<sup>1</sup> In Fig 1, smoke from the burning cigarette which is protected from drafts by a jacket *h*, is passed by way of the ammonia tube *g*, through 3 cc of water, saline or Ringer solution, at room temperature (21 to 30°C), and at 37.5°C maintained by artificial means, by immersion of cylinder *f*, in a water bath A few experiments were performed using light mineral oil as a solvent for the smoke By means of a filter pump, tube *e* sucks air through the cigarette and through the funnel-stopcock system *b* The tipping bucket *a* empties water into the funnel, temporarily preventing ingress of air This sucks air first through the cigarette and then the solution The water drops to the bottom of the cylinder and leaves through tube *d*, by gravity When the tube *b*, becomes free from water, all the air sucked through the system goes through this tube, and none through the cigarette This cycle yields a puff, 100 of which averaged 24.3 cc with the limits of 21 to 28 cc, and which requires 15 seconds to complete, 4 seconds of which are taken up by suction through the cigarette It requires 15 minutes or 60 puffs to smoke one cigarette Through each 3 cc of solution is drawn the smoke from 5 cigarettes in order to insure saturation

\* This research was made possible through a grant by Philip Morris & Co, Ltd, Inc

<sup>1</sup> Hirschhorn and Mulinos, *Proc Soc Exp Biol and Med*, 1930, **28**, 168

In a human volunteer 5 mg/kg of ethyl chaulmoograte administered orally in a gelatin capsule was tolerated with some nausea but no emesis, while 10 mg/kg brought about emesis in 63 minutes. Dr. H. I. Cole has told us that he has observed patients tolerant to 5 cc, or about 100 mg/kg of ethyl chaulmoograte after taking this agent orally for some time.

Among the many peculiar pharmacological actions of *Cannabis sativa* is its role in the *Tai-Fong-Chee* oral method of administering chaulmoogrates recommended by Travers<sup>8</sup>. Travers reports a mixture of 2 parts of powdered *Hydnocarpus* nut and 1 part of *Cannabis indica* to be well tolerated by humans. We have found 5 of 5 cats tolerate 200 mg/kg of ethyl chaulmoograte or 100 mg/kg of fluid extract of *Cannabis* is given simultaneously or previously.

**Summary** Evidence is presented indicating that the emetic effect of the chaulmoogrates is central. The action of *Cannabis*, atropine and morphine in abolishing the emetic response in dogs and cats is reported.

## 7629 C

### An Inexpensive Tissue for Biological Testing

F. F. YONKMAN AND ALICE B. RICHARDS (Introduced by A. W. Rowe)

From the Department of Pharmacology, Boston University School of Medicine, and the Evans Memorial of the Massachusetts Memorial Hospitals, Boston

For some time we have been interested in reactions of the iris to various reagents and have observed that a strip of sphincter pupillae of the steer iris affords an excellent preparation for studying the effects of many drugs on unstriated muscle and its innervation. We suggest the adoption of this tissue for pharmacological studies because of its inexpensiveness, certainty and sensitivity of response, availability at any abattoir and its viability even after 3 to 7 hours post mortem. Precautions regarding preparation of iris strips are given elsewhere.<sup>1, 2</sup>

Besides demonstrating the antagonistic relaxing action of various concentrations of atropine against sphincter contraction by physostigmine one is able to produce opposite effects with histamine and

<sup>8</sup> Travers, E. A. O., *Proc. Roy. Soc. Med.*, 1926, **10**, 1

<sup>1</sup> Miller, G. H., *J. Pharm. and Exp. Therap.*, 1926, **28**, 219

- Yonkman, F. F., *J. Pharm. and Exp. Therap.*, 1930, **40**, 195

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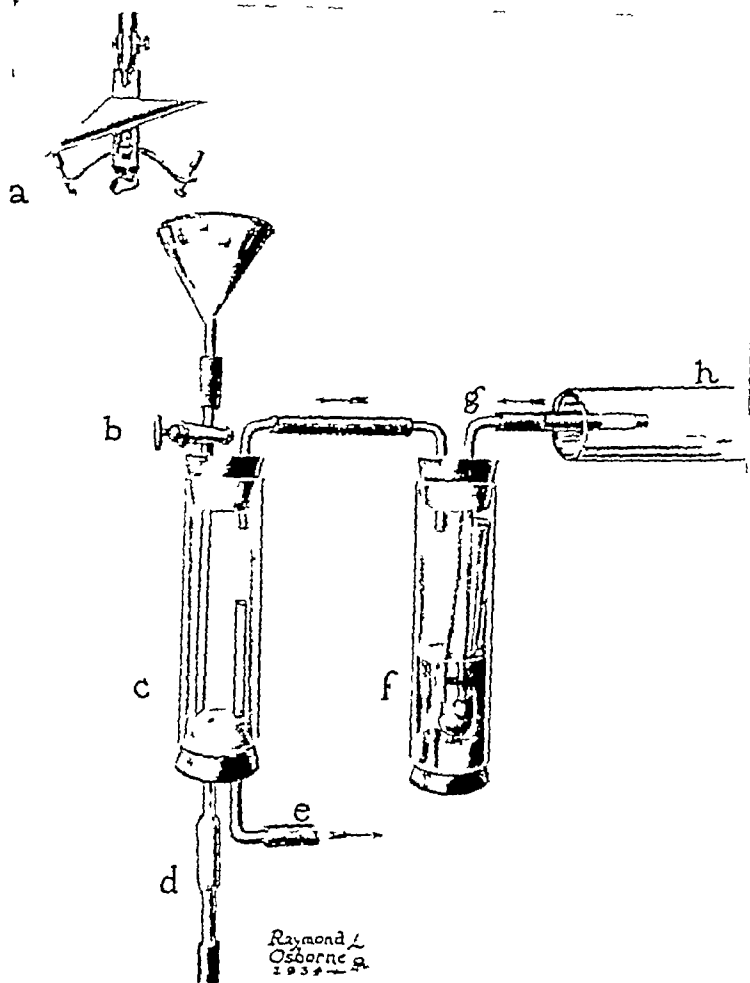


FIG 1

Apparatus used to puff smoke through the 3 cc of fluid in the test tube in jacket

In order to avoid the interference of too many factors which are of necessity inherent in the problem we have limited this first investigation to the influence of the 2 hygroscopic agents usually employed in the manufacture of cigarettes. These are glycerine and di-ethylene-glycol. These hygroscopic agents are deemed necessary to maintain the proper moisture content of the cigarette. It is possible that these chemicals may offer some interference with the combustion of the tobacco, or through their own combustion add quali-

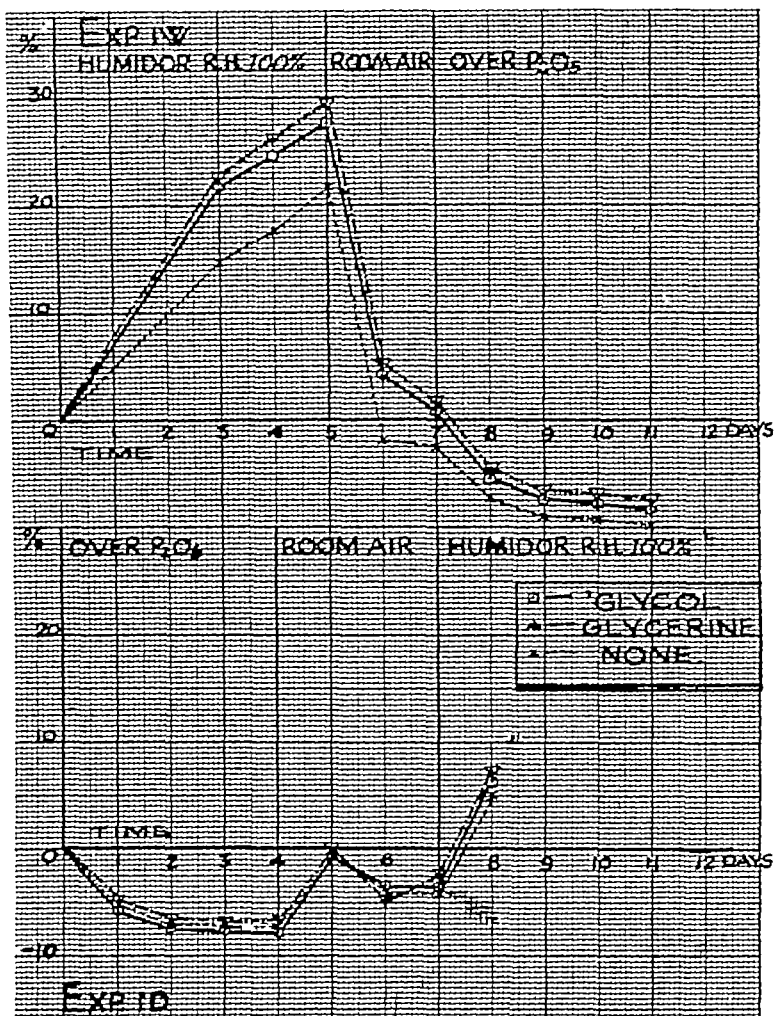


FIG. 2

At 0, 20 cigarettes of each variety were placed under the conditions noted on the chart

ties to the smoke which are not present when the tobacco itself is burned

Through the splendid cooperation of Philip Morris & Co., we obtained the necessary limitation of factors to make the investigation worth while. Under our personal supervision they prepared a batch of tobacco which was treated with the usual casing minus any hygroscopic agent. This was divided into 3 parts, to one was added

2.74% di-ethylene-glycol, to another 3.65% glycerine, and to the third nothing. From each of these, cigarettes were made in the usual manner. The above amounts of hygroscopic agents have approximately equivalent water holding powers, as is shown in Fig. 2 and are those ordinarily used in the manufacture of cigarettes. In addition we obtained some of the same tobacco to which we added subsequently 1, 3 and 5% of each hygroscopic agent.

The cigarettes were smoked under controlled conditions of humidity and also with many variations in the water content of the cigarettes from very low (dried over phosphorus pentoxide), to very wet from standing over water for various periods of time (Fig. 2). However, these variations in the water content of the cigarettes had no demonstrable effect upon the irritating property of the cigarette smoke.

Two to 3 drops of the fluid through which had been sucked the smoke from 5 cigarettes were instilled into the conjunctival sac of each of 3 rabbits. The degree of blepharospasm and the amount of objection by the animal were noted. At 2 minute intervals the condition of the conjunctival mucous membrane was noted, and the edema and redness compared with that of the opposite untreated eye. In Fig. 3, the edema is reported as from plus to 4 plus.

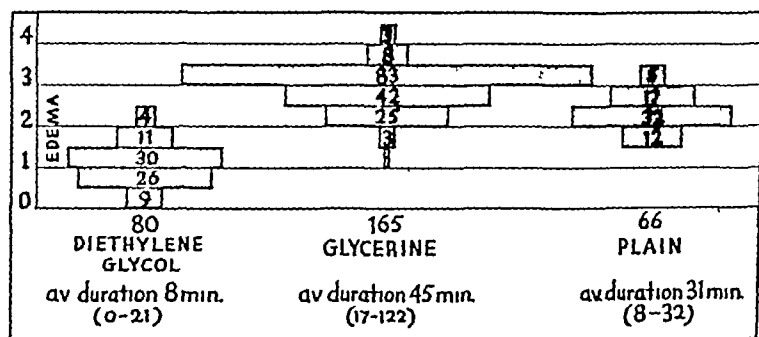


FIG. 3

Frequency curve showing the distribution of the degree of edema in each experiment

**Results** Fig. 3 is a distribution curve of all the experiments performed. It is obvious that the cigarettes which had been made with di-ethylene-glycol as hygroscopic agent proved to be less irritating than those with no hygroscopic agent, and much less irritating than those with glycerine.

The solutions of the cigarette smoke were all acid to litmus, but no attempt was made to determine whether the acidity could account



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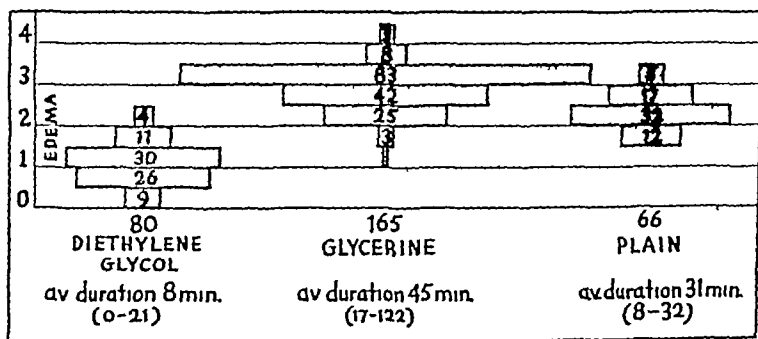


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